Infection and Drug Resistance

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ORIGINAL RESEARCH

The Inhibitory Efficiencies of Geraniol as an Anti-Inflammatory, Antioxidant, and Antibacterial, Natural Agent Against Methicillin-Resistant *Staphylococcus aureus* Infection in vivo

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Introduction: Antibiotics wee widely used as feed additives in animal husbandry. With the increase of drug resistance of bacteria, there is an urgent need to find alternatives to antibiotics. Clinically, methicillin-resistant *Staphylococcus aureus* (MRSA) infections account for about 25% to 50% of *Staphylococcus aureus* infections worldwide. Similarly, it is also one of the pathogens that cause serious animal infections.

Methods: We established a mouse model of systemic infection of MRSA to study the preventive effect of geraniol on MRSA and the immunomodulatory effect of geraniol. The mice in the experiment were injected with geraniol by intramuscular injection and were fed intraperitoneally with minimum lethal dose of MRSA. Then, the survival rate, inflammatory cytokines, oxidative stress factors in serum were measured. These values were used to estimate the bacterial load in different organs and to assess histopathological changes in the lungs, liver and kidneys.

Results: The above-mentioned two ways of using geraniol could prevent MRSA infection in vivo in mice and showed a significant dose–response relationship. In other words, geraniol significantly decreased the concentrations of inflammatory cytokines and oxidative stress factors in MRSA-infected mice. At the same time, the level of glutathione peroxidase also increased in a dose–proportional relationship. In the group of mice treated with geraniol, their superoxide dismutase levels were significantly higher than those in the vancomycin. After treatment with geraniol, the burden of MRSA decreased. No obvious histopathological abnormalities were found in the liver and kidney of MRSA-infected mice. In addition, geraniol improved the inflammatory changes in the lungs.

Conclusion: The results indicated that geraniol was a natural substance that could be used as an anti-inflammatory, antioxidant and antibacterial substance to protect mice from MRSA systemic infection. Generally, the research shows that as a natural medicine, geraniol has broad potential in the development and application of antibiotic substitutes.

Keywords: MRSA, geraniol, antibacterial activity, inflammatory cytokines, in vivo

Introduction

The discovery of penicillin is hailed as one of the greatest inventions, it protects human health to a great extent. As more and more antibiotics are developed, it was no longer limited to the treatment of human diseases. Antibiotics are widely used for disease prevention and growth promotion in conventional livestock and poultry production.[1] The global antibiotic consumption in food animal production was

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conservatively estimated at 63,151 tons/year at present.[2] Concurrent with the success of antibiotics for treating infections, the emergence and rapid dissemination of antibiotic-resistant bacteria poses substantial risks for human health. In addition, the rate of development of antibiotics was not positively correlated with the rate of antibiotics resistance. In particular, the emergence of multidrug-resistant strain methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to almost all kinds of clinical antibiotics including most of β-lactams, aminoglycosides, fluoroquinolones, macrolides, etc. has brought more difficulties to the infection treatment caused by MRSA.[3] Studies have revealed that animals and animal-derived foods have become a new repository for MRSA, and found that there is a transmission chain of MRSA between humans and animals.[4] In view of the difficulties and challenges facing the current diagnosis and treatment of MRSA infection, it is necessary to carry out alternative therapy and the prevention of MRSA infection.

Alternative antibiotic products can help reduce the need for antibiotics by preventing and controlling infectious diseases in animal populations, which are critical to the future success of animal agriculture, and will not cause the trouble of drug resistance.[5] Recently, pure compounds from natural products are gaining acceptance as potentially promising complementary and alternative medicines for the treatment of various diseases. Studies have shown that essential oils have the effect of resisting multi-drug resistant bacteria, including *Candida tropicalis, Candida parapsilosis, Candida* and trichomonas.[6–8]

Geraniol is one of the representatives of acyclic isoprenoid monoterpenes, which can be separated from the essential oils of aromatic plants, including *Cinnamomum tenuipilum, Valeriana officinalis* and several other plants.[9] It was widely used in the food industry and medicine. Researchers have found that geraniol has a variety of pharmacological effects, including antitumor, anti-inflammatory, antioxidative, and antimicrobial activities.[10–12] After preliminary research, it was found that geraniol has effective anti-MRSA activity in vitro; therefore, geraniol has the potential to become an alternative to antibiotics.[13]

Materials and Methods

Animals

Specific pathogen-free (SPF) strains of both male and female KM mice (weight: 20 ± 2 g) were obtained from the Chengdu Institute of Biological Products Co., Ltd., Chengdu Medical College, China. The animals were housed in cages (five animals of the same sex per cage) in ambient temperature (25 ± 2°C) and relative humidity (50 ± 10%). All animals were provided with standard food and water *ad libitum*. The mice were acclimatized in the laboratory environment for three days before the initiation of the experiment. Animal experiments were conducted under the principles of good laboratory animal care and performed in compliance with the Animal Ethics Review Committee of Chengdu Medical College, and this committee also approved the experiments.

Reagents and Strains

Geraniol and vancomycin were purchased from Sigma (St Louis, USA) and the oil for injection was obtained from Zhejiang Tian Yu-Shan Pharmaceutical Oil Co., Ltd (Zhejiang, China). The MRSA strain was obtained from the American Type Culture Collection (ATCC 43300).

Establishment of the Infection Model

MRSA (ATCC 43300) was cultured on nutrient agar at 37°C to the exponential phase and has been diluted with physiological saline to five different concentrations. The KM mice were randomly allocated into six experimental groups (n=10 in each group). The animals were injected intraperitoneal (IP) either with physiological saline (control) or various concentrations of the MRSA suspension. When all the mice in the control group were alive after 72 h, the mortality rate in each of the other groups was observed for determining the minimum lethal dose (MLD) of MRSA.

Administration of Drugs

Two modes of administration were used: gavage and intramuscular (IM) injection. The different dosages of drugs are shown in Figures 1 and 2.

Administration of Muscular Injection

The mice were weighed and randomly allocated into fifteen experimental groups (n = 10 in each group). The daily therapeutic schedule was implemented for three consecutive days. In the healthy control group and infection model group, the mice were administered intramuscular (IM) injection with the same volume of physiological saline. The mice in the positive control group were injected with vancomycin (IM). While in the experimental groups, the mice were injected with different concentrations of geraniol (IM) once per day for the three consecutive days

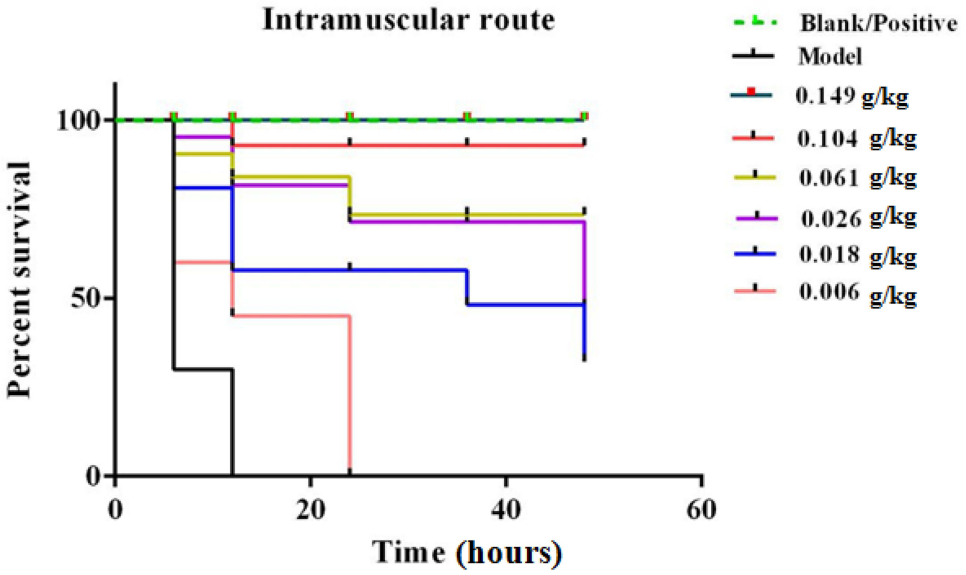

Figure 1 Inhibition of in vivo MRSA activity via administration of geraniol by intramuscular route.

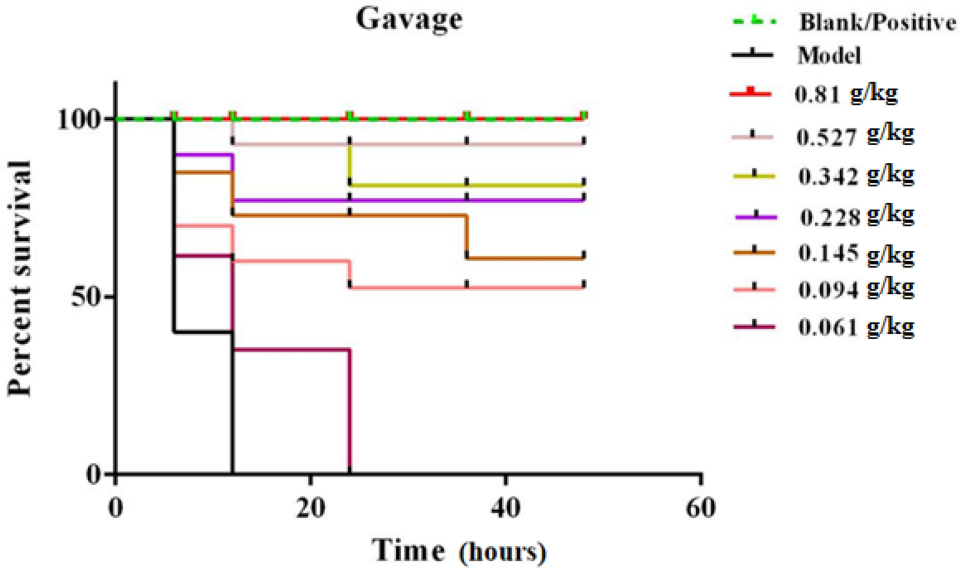

Figure 2 Inhibition of in vivo MRSA activity via administration of geraniol by gavage.

(Figure 1). Except for the healthy control group, all the mice in the other groups were injected IP with the MLD of MRSA after the 3rd day. The survival rate of the mice after seven days of infection was recorded.

Serum Collection

Mice were injected with different doses of geraniol (IM) once per day for three successive days; the 0.149 g/kg, 0.104 g/kg, and 0.061 g/kg dose groups were labelled as high preventative dose (H), medium dose (M), and low dose (L), respectively. Seven days after the mice were infected by MLD of MRSA, the mice were euthanised by the intraperitoneal injection of 20% pentobarbital at 3 mL/kg of body weight, then cervical dislocation. Blood samples were collected from the group of surviving mice. Serum samples were separated from blood by centrifugation for further biochemical studies.

Estimation of Cytokines and Oxidative Factors

The levels of the three main inflammatory cytokines [tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and

interleukin-1 beta (IL-1β)] and the four oxidative factors [glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), and hydroxyl radicals (OH^-)] in the serum were estimated by ELISA technique (Jiancheng Bioengineering Institute, Nanjing, China).

Estimation of the MRSA Load

Blood, livers, and lungs, from the surviving animals of geraniol injected groups, were collected and homogenised for 12 h. The homogenates were serially diluted in phosphate-buffered saline and inoculated on oxacillin-containing soy agar plates that were incubated at 37°C overnight and then the numbers of bacterial colonies were counted.

Histopathological Examinations

At the end of the 7th day of treatment, the different organs (lungs, livers, and kidneys) were obtained from the negative and positive control groups, and from the 0.149 g/kg geraniol-treated groups, and were fixed in 4% formaldehyde solution for 24 h. The specimens were then transferred to a new fixation solution and stored at 4°C. The samples were sent to Chengdu Lilai Biotechnology Co., Ltd. (Chengdu, China), where they have been dehydrated, embedded, sectioned, and stained for further histopathological analysis.

Acute Oral Toxicity Study in Mice

Acute oral toxicity test was conducted according to the guidelines of acute toxicity studies for the Organization for Economic Cooperation and Development (OECD 425) and traditional Chinese medicine. Mice were divided into 2 groups of 10 mice each and weigh (5 males and 5 females). The acute toxicity test was decided to use a single dose of 2500 mg/kg body weight/oral geraniol, the mice were fasted for 12 hours before giving geraniol. Give normal water and diet after 4 hours of administration. The mice were observed the behavior and the number of death within 24 hours, such as spontaneous motor activity, excretion, vocalization, etc. The body weight and food consumption were monitored on Days 0, 3, 6, 9, 12 and 14.

Statistical Analysis

Karber's method was used to test the median effective dose (ED_{50}) of geraniol (IM and gavage) in KM mice. One-way analysis of variance (ANOVA) was used to analyse the continuous variables. All analyses were performed using SPSS 19.0. P values of <0.05 and $P<0.01$ were regarded as significant and very significant, respectively.

Results

Efficacy of Geraniol in Preventing MRSA Infections

Geraniol administered via IM route and gavage showed efficiencies in preventing MRSA infection in vivo (Figures 1 and 2) in dose-dependent manners. At doses 0.149 g/kg (IM) and 0.810 g/kg (gavage), the survival rate of the MRSA-infected mice was 100%. By Karber's method, the ED_{50} was calculated to be 0.030 g/kg (IM) and 0.197 g/kg (gavage).

Effects of Geraniol on the Inflammatory Mediators and Oxidative Factors

As shown in Figure 3, the levels of the three inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the MRSA-infected group were significantly higher than that in the negative control group ($P<0.01$). After treatment with geraniol, the levels of IL-1β, IL-6, and TNF-α were decreased in a dose-dependent manner. Similarly, the IL-6 and TNF-α level in the high-dose treatment group was restored to the normal range. They were similar to the negative control group, without showing any significant difference between them.

In the positive control group, the IL-1β ($P<0.01$), IL-6 ($P<0.01$), and TNF-α ($P<0.05$) levels were significantly reduced as compared to the treatment groups. Similarly, IL-1β level in all treatment groups, IL-6 level in the high- and medium-dose treatment groups, and TNF-α level in the high-dose treatment group were significantly lowered ($P<0.01$) as compared to the MRSA-infected group. The IL-1β and TNF-α level in the high- and medium-dose treatment groups was similar. The IL-6 level in all treatment groups, and the IL-1β and TNF-α level in the low-dose treatment groups were significantly lowered ($P<0.01$) compared to the positive control group. These results indicated that geraniol could dramatically reduce the levels of IL-1β, IL-6, and TNF-α in the MRSA-infected mice similar to vancomycin.

As shown in Figure 4, the levels of GSH-Px and SOD were significantly decreased ($P<0.01$), and the levels of MDA and OH^- were significantly increased ($P<0.01$) in the MRSA-infected group, which were all reversed by the administration of geraniol, in a dose-dependent manner. There was no significant difference in the GSH-Px level between the negative and positive control groups. As compared to the negative control group, the SOD level was significantly decreased ($P < 0.01$), and the MDA and OH^- levels were significantly increased ($P<0.01$) in the positive control group.

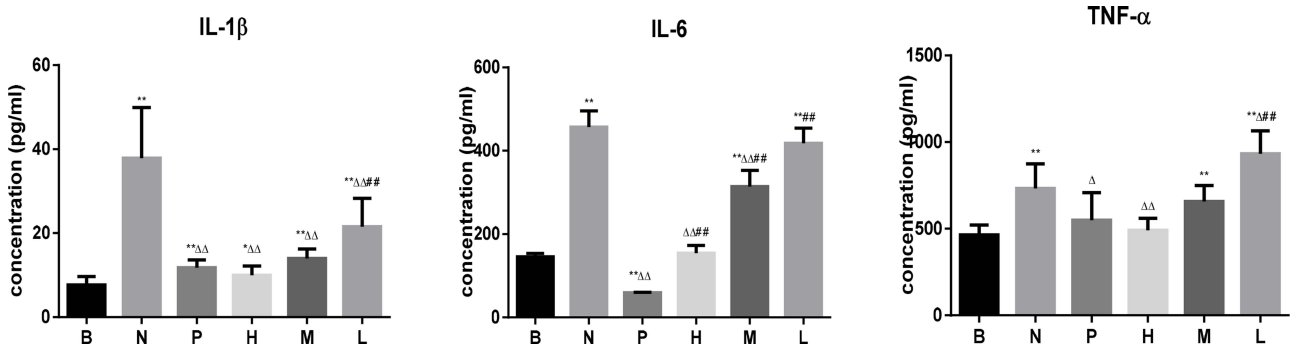

Figure 3 Effect of geraniol on inflammatory factors in MRSA-infected blood. B: control group, N: model group, P: positive control group, H: high dose group, M: medium-dose group, L: low dose group. *<0.05, **<0.01 represents a significant and very significant difference among the groups compared to control, respectively. $^{\Delta}$<0.05, $^{\Delta\Delta}$<0.01 represents a significant and very significant difference among the groups compared to the model, respectively. $^{\#\#}$<0.01 represents very significant difference among the groups compared to the positive control.

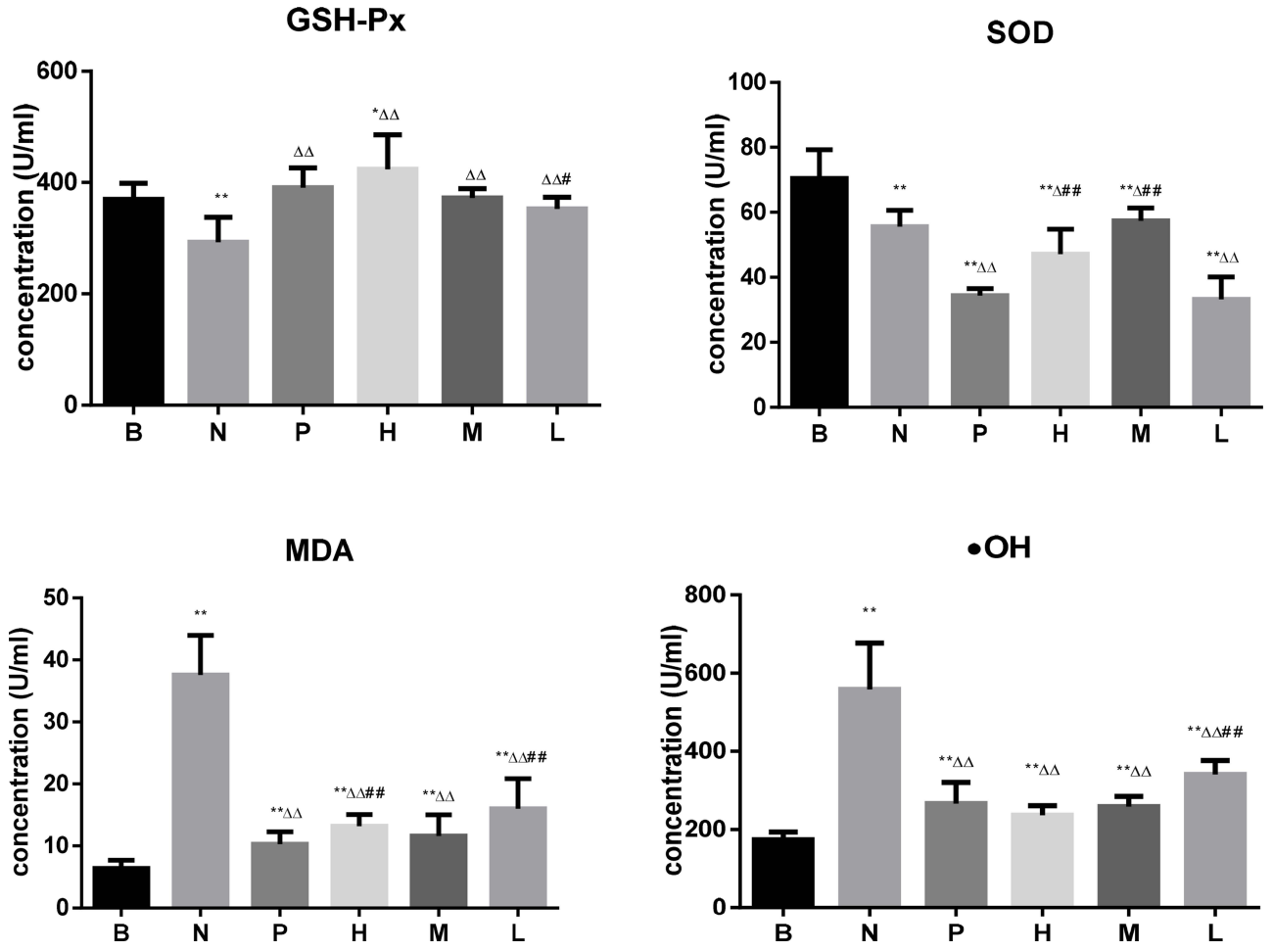

Figure 4 Effect of geraniol on oxidative factors in MRSA-infected blood. B: control group, N: model group, P: positive control group, H: high dose group, M: medium-dose group, L: low dose group. *<0.05, **<0.01 represents a significant and very significant difference among the groups compared to control, respectively. $^{\Delta}$<0.05, $^{\Delta\Delta}$<0.01 represents a significant and very significant difference among the groups compared to the model, respectively. $^{\#}$<0.05, $^{\#\#}$<0.01 represents a significant and very significant difference among the groups compared to the positive control, respectively.

A GSH-Px level in the high- and low-dose treatment groups was restored to the normal range and was similar to the negative control group.

The GSH-Px level in the high-dose treatment group and the SOD, MDA, and OH^- levels in all treatment groups were significantly different compared to the control

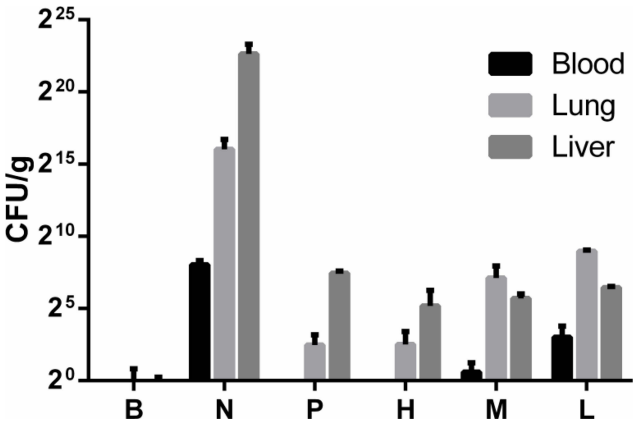

Figure 5 Bacteria loads of organs (blood, lung, and liver) in control and geraniol injected groups. B: control group, N: model group, P: positive control group, H: high dose group, M: medium-dose group, L: low dose group.

group (P<0.01). There were significant differences in the GSH-Px, SOD, MDA, and OH^- levels (P<0.01) between the positive control group and the MRSA-infected groups.

Similarly, GSH-Px level was significantly higher (P<0.01), and MDA and OH^- levels were lowered considerably (P<0.01) in all treatment groups as compared to the MRSA-infected group. The GSH-Px and OH^- levels in the high- and medium-dose treatment groups, SOD level in the low doses group, and MDA level in the medium-dose treatment group were similar to the positive control group. These results indicated that geraniol could effectively regulate the levels of GSH-Px, MDA, and OH^- in the MRSA-infected mice similar to vancomycin.

Effects of Geraniol on the MRSA Load

Counting the bacterial colonies in blood, lung, and liver homogenates showed dramatically low colony-forming units in the geraniol-injected and positive control groups. Consistent with this finding, the mice in the geraniol-treated group exhibited improved symptoms as compared to the negative control group (Figure 5).

Effects of Geraniol on the Histopathological Changes

The MRSA-infected mice showed inflammatory changes in the lung tissues. Neutrophilic infiltrations in the interstitial lung cells and a few collapsed alveolar cavities were detected (Figure 6). There were, however, no apparent histopathological abnormalities in the livers and the kidneys (Figures 7 and 8).

The inflammatory changes in the lungs were significantly mitigated by geraniol (Figure 9). There were no obvious neutrophilic infiltrations in the interstitial cells, and the alveolar structures were found to be clear. These results indicated that geraniol could effectively improve lung inflammation in MRSA-infected mice.

Acute Toxicity Study

The geraniol at a dose of 2500 mg/kg did not cause mortality or any significant signs of acute toxicity in the mice observed for short period of time (24 h) and continued observation to 14 days. All animals looked bright and active. Meanwhile, their fur color was clean and uncluttered. There were no significant differences in body weights and food consumption between the geraniol administrated group and the control group.

Discussion

Now antibiotics are not only used to treat infectious diseases, it was sometimes given to food animals at low doses in order to promote faster growth.[14] The widespread use of antibiotics also brings more and more problems, include destroy the microbial composition of soil and water,[15] antibiotic residues in food,[16] increased antibiotic resistance,[17] etc.

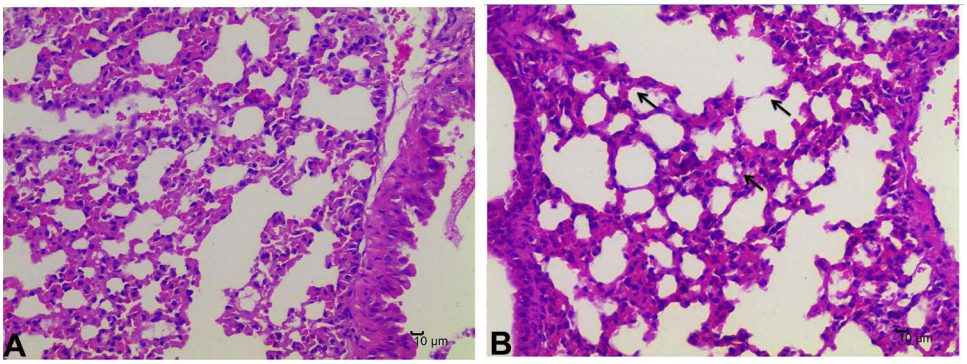

Figure 6 The pathological changes of the lung tissue of MRSA-infected mice. (**A**): control group, (**B**): model group.

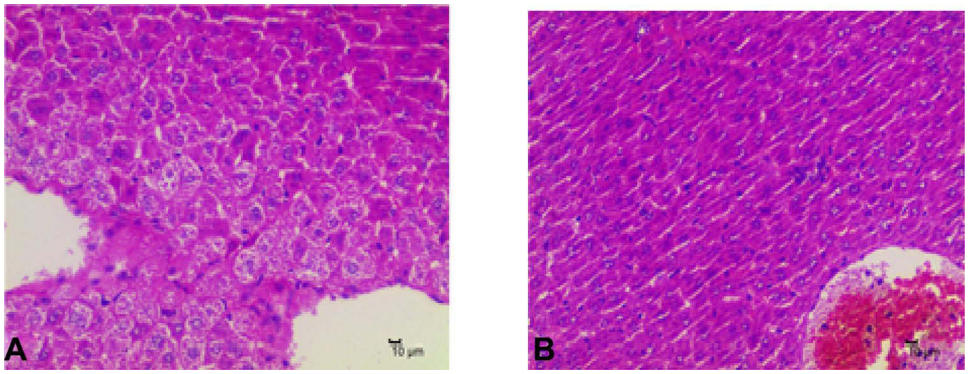

Figure 7 The pathological changes of the liver tissue of MRSA-infected mice. (**A**): control group, (**B**): model group.

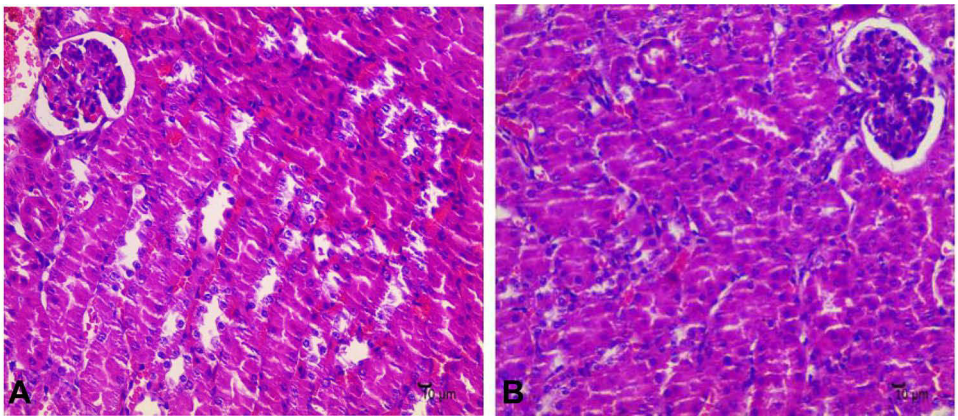

Figure 8 The pathological changes of the kidney tissue of MRSA-infected mice. (**A**): control group, (**B**): model group.

According to the Centers for Disease Control and Prevention report, nearly one in seven patients contracting severe MRSA infections die.[18] With the spread of bacteria, MRSA has been found not only in human infectious diseases, but also in food and animal husbandry[19,20] (included cow mastitis caused by MRSA infection),[21] it caused great economic losses. Vancomycin, a glycopeptide antibiotic, was used for the treatment of MRSA infections for many years. As of 2019, the detection rate of VRSA in Asia was 1.3%, and the detection rate in Europe was 1.1%.[22] New antibiotics like linezolid are used for VRSA infections, but the prevalence of linezolid resistance has become increasingly serious, including *Enterococcus, Staphylococcus squirrel* and *Streptococcus*.[23–25]

Geraniol is well known for its antifungal, antioxidant, anticancer, anti-nociceptive, as well as antidepressant-like effects.[26,27] At the same time, the essential oils containing geraniol are used in the clinical setting as an anti acne, against pityriasis versicolor, candida and in eye infection diseases.[28–31] Herein, we have successfully evaluated geraniol's therapeutic activity in an in vivo MRSA-infected mouse model. Geraniol administered by the IM route and gavage exhibited dose-dependent protective activities against MRSA similar to vancomycin. At doses 0.149 g/kg (IM) and 0.810 g/kg (gavage), the survival rate of MRSA-infected mice was 100%. Geraniol able to permeate directly from the bloodstream to the central nervous system following its oral administration to rats, reaching detectable amounts in the CSF. At the same time, the absorption rate (30-minute peak blood concentration) and absorption (92% absolute bioavailability) test results show the high bioavailability of geraniol.[32] This makes it possible for geraniol to be used as a drug for research and development.

The *S. aureus*-infected host produces numerous cytokines as a part of the immune defence mechanism. The immune defence mechanism of the MRSA-infected mice was found to be significantly weaker compared to the negative control group. During an inflammatory response, excessive production

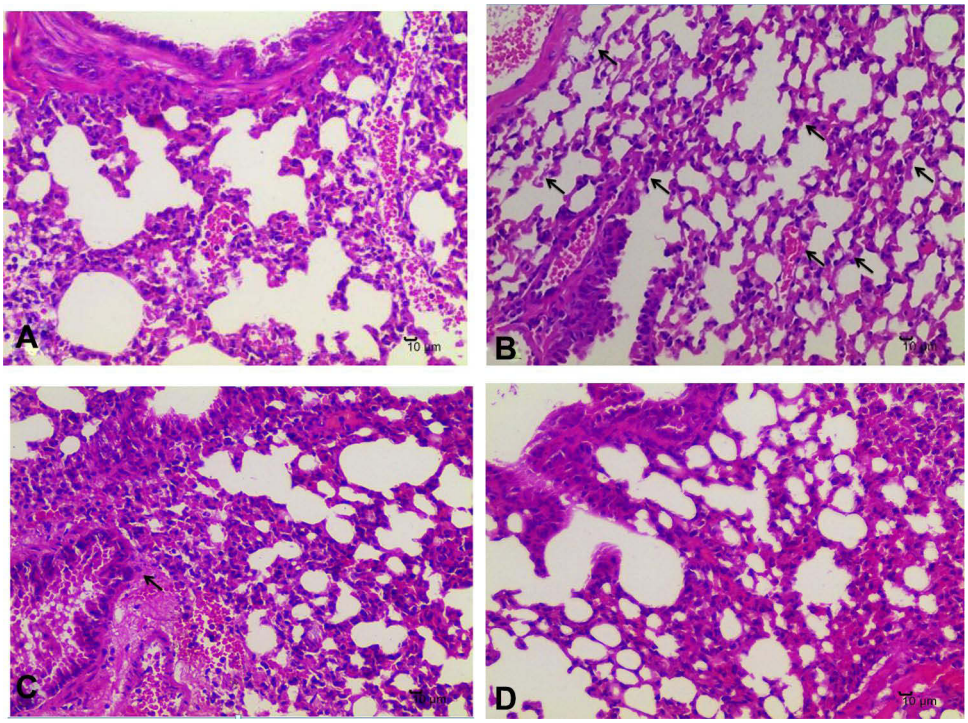

Figure 9 Effect of geraniol on lung tissue of MRSA-infected mice. (**A**): control group, (**B**): model group, (**C**): positive control group, (**D**): experimental group.

of cytokines, such as IL-1β, IL-6, and TNF-α, can lead to multiple organ dysfunction syndromes, septic shock, and death.[33,34]

S. aureus can cause different diseases including, septicaemia and can induce a systemic inflammatory response.[35–38] As MRSA-induced septicaemia is directly related to the exaggeration of the host systemic inflammatory response, the downregulation of the inflammatory pathways is significant as a therapeutic strategy. Our study showed that the levels of IL-1β, IL-6, and TNF-α were significantly increased in the MRSA-infected mice. In contrast, the levels were decreased upon treatment with geraniol in a dose-dependent manner.

Reactive oxygen species (ROS) is produced from different oxidation-reduction reactions in the healthy cells, such as the production and clearance of hydroxyl radical (OH^-) in a dynamic equilibrium state. When endogenous or exogenous harmful stimuli challenge the system, oxidative stress (OS) can result from an increased ROS production.[39] Oxidative damage to DNA and abnormal protein expression can be caused by OS, leading to different diseases.[40] Malondialdehyde (MDA), a small molecule produced during the termination of the lipid peroxidation reaction chain, can seriously damage the cell membrane and causes cellular swelling and necrosis.[41] In this study, the levels of MDA and OH^- were significantly increased in the MRSA-infected mice, whereas their levels were reversed with geraniol treatment.

In a normal physiological environment, the body has an inherent enzymatic antioxidant system that protects against the harmful effects of ROS, including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).[42,43] SOD is one of the essential antioxidant enzymes present in the cells, which can scavenge oxygen-free radicals to protect the cells from damage.[44] GSH-Px is widely utilised by the cells to convert toxic peroxide compounds to non-toxic hydroxyl compounds.[45] Our study showed that the levels of GSH-Px and SOD were significantly reduced in the MRSA-infected group, which were reversed with geraniol treatment. The SOD level did not return to a normal range in the geraniol-treated group, although it was significantly higher than that in the vancomycin-treated group.

Different pathological changes in the lungs were observed in a model of hematogenous pneumonia established by injecting the infective organism through the tail vein.[46,47] Herein, the MRSA-infected mice demonstrated inflammatory changes in the lung tissues, although there were no apparent histopathological abnormalities in the livers and the kidneys. The inflammatory changes in the lungs were ameliorated by geraniol. These results were consistent with Li et al,[48] who revealed that the administration of geraniol

inhibited the inflammatory responses and apoptosis in the lung.

Conclusions

Our study implicates that geraniol exhibits strong preventive potential against MRSA-induced oxidative and inflammatory events in the lung while no apparent histopathological abnormalities were observed in the livers and the kidneys. Histopathological observations showed that alleviative effect of geraniol is due to its anti-oxidative and anti-inflammatory potential. At the same time, the acute toxicity test shows that the geraniol has a large safety range, and the experimental animals have no toxicity.

Geraniol administered via I.M route and gavage exhibited a dose-proportional protective activity against MRSA infection in vivo. The protective mechanism of geraniol has mainly attributed to the regulation of the inflammatory cytokines (IL-1β, IL-6, and TNF-α) and oxidative factors (GSH-Px, MDA, and OH^-) coupled with amelioration of the inflammatory changes in the lung. Therefore, geraniol has a promising potential as feed additives to be developed and applied for the prevention of MRSA infection.

Transparency Declaration

All authors have read and approved the manuscript.

Acknowledgments

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Disclosure

Min Dai and Fenghui Sun are co-correspondence authors for this study. The authors report no conflicts of interest in this work.

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