

Inhibitory Activity of Ethanol Extract from *Artemisia argyi* on a Clinical Isolate of *Staphylococcus aureus*

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Received 4 October 2014; revised 19 November 2014; accepted 30 November 2014

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Abstract

As a traditional herbal medicine for the treatment of many disorders, *Artemisia argyi* has shown many valuable bioactivities, but little is known about its effect on *Staphylococcus aureus*. In this study, the growth, the biofilm formation and the pathogenicity of *S. aureus* cultivated with or without ethanol extract of *A. argyi* were tested using microtitre plate assay, Confocal Laser Scanning Microscope (CLSM) system and mice infection assay. Results showed that the growth and the biofilm formation of *S. aureus* in the test group with ethanol extract of *A. argyi* were significantly lower than those of the control group without ethanol extract of *A. argyi*. With CLSM system we could observe that the biofilm structure of the test group had looser and less biomass compared with the control group. After infection of *S. aureus*, the survival of mice in test group that were given 0.2 mL 100 mg/mL ethanol extracts of *A. argyi* was higher than the control group. Histopathological analyses showed that the tissue damage of mice in test group was less than that in control group. These results suggested that ethanol extract of *A. argyi* had inhibitory effect on *S. aureus* and could protect mice from death induced by *S. aureus* infection.

Keywords

Artemisia argyi, *Staphylococcus aureus*, Biofilm Formation, Pathogenicity

1. Introduction

Artemisia argyi, the Chinese mugwort, is an aromatic plant in the genus *Artemisia*, which is native to China, Japan, and far-eastern Siberia. It is an herbaceous perennial plant with a creeping rhizome known in Chinese as

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aicao and in Japanese as gaiyou. It has been used as a traditional herbal medicine for the treatment of microbial infections, inflammatory diseases, hepatitis, diarrhea, cancer, malaria, circulatory system and metabolism disorders [1]-[3]. This plant contains many valuable compounds such as coumarins, flavonoids, glycosides, polyacetylenes, sterols, monoterpenes, triterpenes, essential oils and sesquiterpene lactones, some of which have presented many bioactivities such as antiulcer [4], antidiabetic [5], anti-inflammatory [6], and anticancer [2] activities. However, there is little information available about its effect on *Staphylococcus aureus*.

S. aureus is an important opportunistic pathogen leading cause of nosocomial- and community-acquired infections worldwide, which can cause a variety of infectious diseases, from relatively benign skin infections to potentially life-threatening systemic disorders such as osteomyelitis and endocarditis [7]. *S. aureus* has the ability to form biofilms on the surface of many tissues and implants in humans, which provide a physiological niche to protect bacteria from the host immune response and antibacterial agents. The ability to form biofilm is an important aspect of *S. aureus* pathogenesis to cause persistent and chronic bacterial infections [8]-[11]. Therefore novel strategies that target biofilm-associated infections caused by *S. aureus* will be considered as future therapies [12].

In this study, we investigated the antibacterial activity of the ethanol extract of *A. argyi* to *S. aureus* *in vivo* and *in vitro*. Results show that the ethanol extract of *A. argyi* has inhibitory effect on the growth, the biofilm formation and the pathogenicity of *S. aureus*, which may provide clues for therapy in *S. aureus* infection.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

S. aureus strain 140,328 used in this study were provided by clinical laboratory of the First Affiliated Hospital of Anhui Medical University, which is isolated from the blood of a patient undergoing hemodialysis and identified by VITEK automatic analyzer. *S. aureus* 140,328 pGFP was obtained by transformation of plasmid pGFP that carrying the fluorescent protein to strain 140,328. These stains were grown at 37°C in tryptic soy broth (TSB) medium (BD) or TSB with chloramphenicol (15 µg/mL). Medium was supplemented with 3% sodium chloride or 0.5% glucose when necessary.

2.2. Production of Ethanol Extracts of *A. argyi*

A. argyi was purchased from the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine. It was crushed into powder with crushing apparatus and was suspended in 5 mL 80% ethanol solution per gram and soaked for 24 h at 37°C. The supernatant was collected and the residue was added in 2.5 mL 80% ethanol solution per gram and this step was repeated another time. All these supernatants were mixed into 100 mg/mL concentration extract, which was diluted with 80% ethanol to 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL concentration.

2.3. Antimicrobial Activity

Colonies of *S. aureus* 140,328 were picked into a tube contains 2 mL of TSB medium and cultivated at 37°C shaking 200 rpm for overnight. The second day, this overnight cultures were inoculated into fresh TSB medium and was diluted to a final concentration of OD₆₀₀ 0.05, which was dispensed into 96-well plates (Corning, Steuben, NY) containing serial dilutions of ethanol extracts of *A. argyi* (10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0 mg/mL). At the same time 1 µg/mL, 0.8 µg/mL, 0.7 µg/mL, 0.6 µg/mL and 0.4 µg/mL vancomycin was used as standard antibiotic control. Plates were incubated at 37°C for 12 h and then ten-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through seven microfuge tubes containing 0.9 mL of TSB. 0.1 mL dilutions were dropped on LB agar plates and viable colonies were counted via their colony-forming units (CFU) on TSB agar plates and lgCFU/mL were calculated after incubated at 37°C for 24 h. Experiments were repeated three times with four parallels.

2.4. Biofilm Formation in Microtitre Plate Assay

Biofilm formation under static conditions was determined by the microtitre plate assay based on the method described previously [12]. Briefly, the overnight cultures were made at a 1:100 dilution using fresh TSB contain-

ing 0.5% glucose. The diluted cell suspension was inoculated into flat-bottom 96-well polystyrene plates (Costar 3599, Corning Inc., Corning, NY), 200 μ l each well containing serial dilutions of ethanol extract of *A. argyi* (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0 mg/mL). The plates were incubated at 37°C for 12 h and wells were rinsed gently with water five times to remove non-adherent cells. Subsequently, the plates were stained with 0.5% crystal violet for 15 min, and then rinsed again with water to remove unbound stain. After that, the plates were dried, and the optical density at 560 nm (OD_{560}) was determined with an enzyme-linked immunosorbent assay reader (BioTek Elx800, USA) in a 5 \times 5 scan model.

2.5. Observation of Biofilm Formation in Laser Scanning Confocal Microscope System (CLSM)

Overnight cultures of *S. aureus* 140328pGFP, which was transformed with the GFP plasmid for fluorescence detection from *S. aureus* 140328, was inoculated at a 1:100 dilution in fresh TSB containing 0.5% glucose and 15 μ g/mL chloramphenicol to maintain plasmid selection into glass bottom culture dishes (35 mm petri dish, 10 mm microcell, MatTek, USA), 1 mL each dish. The experimental group contained 2.5 mg/mL ethanol extracts of *A. argyi*. Dishes were incubated at 37°C for 12 h and were rinsed gently with water five times to remove non-adherent cells. CLSM was performed on a Zeiss LSM710 collected at 1 μ m intervals. Selected confocal images stood for similar areas of interest and each confocal experiment was repeated four times. The confocal images were acquired from Zeiss ZEN 2010 software package (Carl Zeiss, Jena, Germany) and the three-dimensional biofilm images were rendered with Imaris 7.0 (Bitplane, Zurich, Switzerland).

2.6. Mouse Infections

Male BALB/c mice were purchased from Shanghai Experimental Center, Chinese Science Academy (Shanghai, China), and housed in isolated cages in an animal facility. *S. aureus* strain 140,328 was grown to early log phase OD_{600} 0.4 - 0.6. Cells were collected by centrifugation at 4000 g and washed twice with sterile PBS and then diluted with sterile PBS and adjusted to OD_{600} 0.5. Viable staphylococci were counted via their CFU on TSB agar plates in order to quantify the infectious dose. Male BALB/c mice that were 5 - 6 weeks old were infected with 0.1 mL OD_{600} 0.5 of *S. aureus* via a lateral tail vein injection and monitored daily for death. Starting from the day of infection, the infected mice were divided randomly into three groups. Every group contained 12 mice and administered by gavage daily. Mice of group c were given 0.2 mL 80% ethanol, mice of group t were given 0.2 mL 100 mg/mL ethanol extracts of *A. argyi*, and mice of group v were given 0.2 mL vancomycin (10 μ g/mL) by gavage. After 7 days, mice that still live were killed and their organs were removed and histopathological analyses were manipulated by the pathological department of the First Affiliated Hospital of Anhui Medical University. The experiment protocol is in accordance with the standard animal welfare guideline, and the permission was obtained from Ethics Committee.

2.7. Statistical Analysis

The data was analyzed using statistical software SPSS 13.0 by a one-way ANVONA method, the test results were (mean \pm standard deviation). The paired *t* test was used for statistical comparisons between groups. The level of statistical significance was set at a P value of ≤ 0.05 .

3. Results

3.1. Inhibitory Effect of Ethanol Extract of *A. argyi* on *S. aureus*

The antibacterial activity of the ethanol extract of *A. argyi* to *S. aureus* in vitro was confirmed by antibacterial assay. After exposure to the extract of *A. argyi* for 12 h at 37°C, the growth of *S. aureus* was affected obviously, which was presented by the viable counts on TSB agar plate after cultivated for 24 h at 37°C. The viable counts of bacteria of the test group containing ethanol extract of *A. argyi* were decreased significantly compared with the control group without the extract of *A. argyi*, and the reduce extent was positively correlated with the concentration of the ethanol extract of *A. argyi* (Figure 1(A)). The viable counts of *S. aureus* cultivated with 1.25 mg/mL concentration of the ethanol extract of *A. argyi* were almost 100 times lower than that cultivated without the ethanol extract of *A. argyi*. Furthermore, with the increase of the concentration of the ethanol extract of *A.*

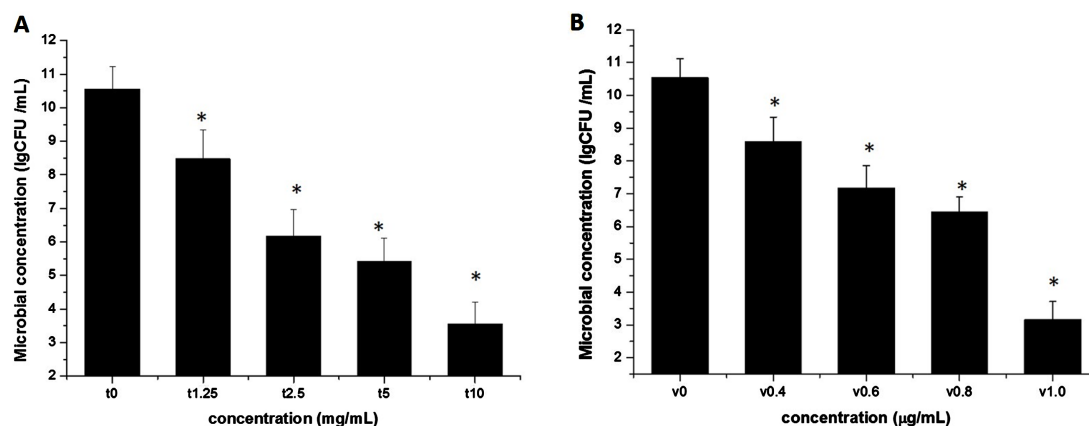


Figure 1. Colony counts (lgCFU/mL) of *S. aureus* 140,328 following 12 h of incubation at 37°C with or without ethanol extract of *A. argyi* (A), with or without vancomycin (B). t0: control group no ethanol extract of *A. argyi*, t1.25: 1.25 mg/mL ethanol extract of *A. argyi*, t2.5: 2.5 mg/mL ethanol extract of *A. argyi*, t5: 5 mg/mL ethanol extract of *A. argyi*, t10: 10 mg/mL ethanol extract of *A. argyi*. v0: control group no vancomycin, v0.4: 0.4 µg/mL vancomycin, v0.6: 0.6 µg/mL vancomycin, v0.8: 0.8 µg/mL vancomycin, v1.0: 1.0 µg/mL vancomycin. *Significantly different from no ethanol extract of *A. argyi* control ($P < 0.05$).

argyi, the viable counts of *S. aureus* reduced more significantly. When cultivated with 10 mg/mL concentration of the ethanol extract of *A. argyi*, the viable counts of *S. aureus* were nearly 10^7 times lower than that without ethanol extract of *A. argyi*. The antibacterial effect on *S. aureus* *in vitro* at this concentration of the ethanol extract of *A. argyi* is equivalent to 1.0 µg/mL of vancomycin (Figure 1(B)). These data show that the ethanol extract of *A. argyi* can inhibit the growth of *S. aureus* *in vitro*.

3.2. Inhibitory Effect of the Ethanol Extract of *A. argyi* on Biofilm Formation of *S. aureus*

To investigate whether ethanol extract of *A. argyi* affects biofilm formation of *S. aureus*, we monitored the biofilm formation of *S. aureus* using a microtitre plate assay. As shown in Figure 2(A), strains of the control group without ethanol extract of *A. argyi* formed strong biofilm after 12 h incubation at 37°C. However, strains of the experimental groups with different concentrations of ethanol extract of *A. argyi* formed weaker biofilm as measured by quantitative spectrophotometric analysis based on OD₅₆₀ after crystal violet staining. Moreover, this discrepancy was more obvious with the increasing of the concentration of the ethanol extract of *A. argyi* when cultivated in TSB containing 0.5% glucose (Figure 2(A)).

In order to directly observe the effect of ethanol extract of *A. argyi* on biofilm formation of *S. aureus*, we used CLSM system and strains that can constitutively express green fluorescent protein GFP. After 12 h incubation at 37°C in TSB containing 0.5% glucose, strains of the control group without ethanol extract of *A. argyi* produced intact and rough biofilms as monitored by CLSM (Figure 2(B)). In contrast, biofilms produced by strains of the experimental group with 2.5 mg/mL ethanol extract of *A. argyi* was undetectable (Figure 2(C)).

3.3. Protective Effect of Death of Mice Induced by *S. aureus* Infection

Mice of control group (group c) that were given 0.2 mL 80% ethanol without the ethanol extracts of *A. argyi* by gavage daily started to die from the day after infected with 0.1 mL OD₆₀₀ 0.5 of *S. aureus* via a lateral tail vein injection (Figure 3(A)). These mice appeared very dispirited and after 7 days, the survival rate of mice in group c was less than 10%. However, mice of test group (group t) that were given 0.2 mL 100 mg/mL concentration of the ethanol extract of *A. argyi* by gavage daily appeared more spirited. The survival rate of mice in group t was about 70%, which is nearly 7 times of group c. Histopathological analyses show that the severity of organ damage in mice of group t was lower than that of group c (Figure 3(B) and Figure 3(C)). There was no much difference of the survival rate between group t and group v that mice were given 0.2 mL 10 µg/mL vancomycin by gavage daily, which suggested that like vancomycin, the extract of *A. argyi* have the ability to protect mice of death induced by *S. aureus* infection.

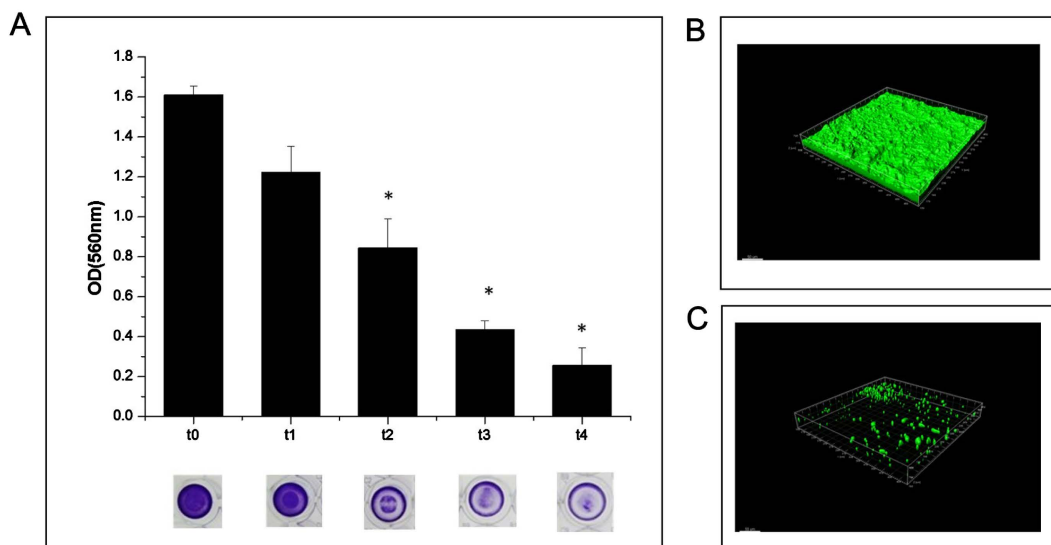


Figure 2. Biofilm formation of *S. aureus* 140328 when cultivated in TSB containing 0.5% glucose in 96-well plates for 12 h at 37°C with or without ethanol extract of ethanol extract of *A. argyi*. The cells that adhered to the plate after staining with crystal violet were measured by OD₅₆₀ (A). Observation of biofilm formation of *S. aureus* 140328pGFP when cultivated in TSB containing 0.5% glucose and 15 µg/mL chloramphenicol in glass bottom culture dishes for 12 h at 37°C without (B) or with 2.5 mg/mL ethanol extract of *A. argyi* (C). t0: control group no ethanol extract of *A. argyi*, t1: 0.625 mg/mL ethanol extract of *A. argyi*, t2: 1.25 mg/mL ethanol extract of *A. argyi*, t3: 2.5 mg/mL ethanol extract of *A. argyi*, t4: 5 mg/mL ethanol extract of *A. argyi*. *Significantly different from no ethanol extract of *A. argyi* control (P < 0.05). Biofilm integrity and GFP fluorescence were monitored by CLSM and the three-dimensional images were rendered with Imaris 7.0.

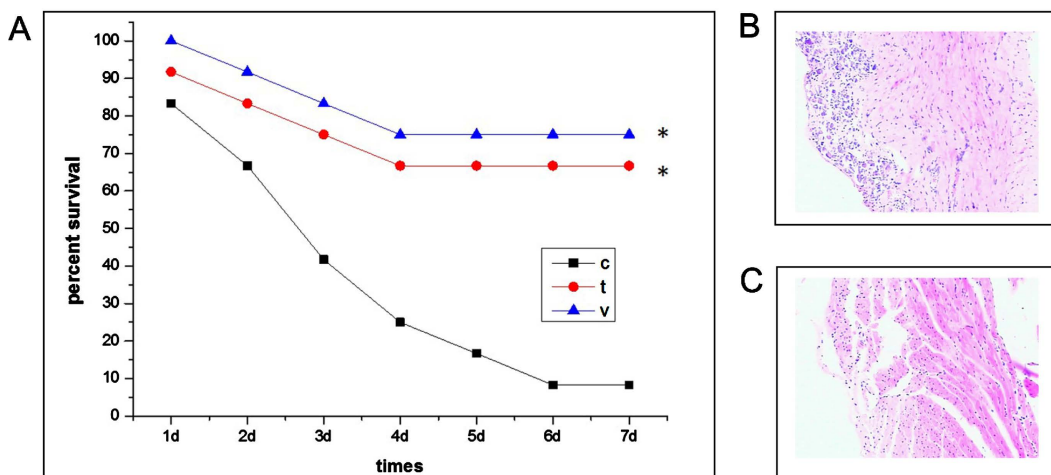


Figure 3. The ethanol extract of *A. argyi* could protect mice of death induced by *S. aureus* infection. (A) The percent survival of mice in 7 days after infected with 0.1 mL OD₆₀₀ 0.5 of *S. aureus* via a lateral tail vein injection and administered by gavage daily with 0.2 mL 80% ethanol (group c ■), with 0.2 mL 100 mg/mL ethanol extracts of *A. argyi*, (group t ●), and with 0.2 mL 10 µg/mL vancomycin (group v ▲). Every group contained 12 mice. *Significantly different from control group (P < 0.05); (B) Histopathology of heart from mice in group t after 7 days administered by gavage daily with 0.2 mL 100 mg/mL ethanol extracts of *A. argyi*; (C) Histopathology of heart from mice in group c after 7 days administered by gavage daily with 0.2 mL 80% ethanol. Magnification ×40.

4. Discussion

S. aureus causes a variety of infections and its biofilm formation capacity plays an important role in chronic infection, which brings serious threat to human health. Especially, in recent years, methicillin-resistant *S. aureus*

(MRSA) has been spread worldwide, rendering the entire β -lactam class of antibiotics ineffective [13]-[15]. So far, vancomycin has been the most reliable therapeutic agent against infections caused by MRSA. Searching for new drugs to inhibit the biofilm formation of *S. aureus* has become a new strategy for clinical infection treatment. Here, we provide evidence that ethanol extract of *A. argyi* has inhibitory effect on the growth, the biofilm formation and the pathogenicity of *S. aureus*, suggesting a potential clue for therapy in *S. aureus* infection.

However, traditional Chinese medicine usually includes many kinds of compound. The antibacterial effect of them often plays roles through a variety of mechanisms, such as disturbance of cell wall synthesis, inhibition of DNA replication and transcription, changes of cell membrane, interference of enzyme system, and disruption of its normal metabolites *in vivo*. This study is only preliminary detection to the effect of ethanol extract of *A. argyi* on *S. aureus*. More specific mechanism involved in this process remains to be further studied in detail.

Considering the valuable compounds of *A. argyi* such as coumarins, flavonoids, glycosides, polyacetylenes, sterols, monoterpenes, triterpenes, essential oils and sesquiterpene lactones, which presented bioactivities in general are fat soluble and could be extracted by 80% ethanol. However, which is the particular ingredient of *A. argyi* that inhibits the growth, the biofilm formation, and the pathogenicity of *S. aureus* and how to inhibit are still unknown. The specific mechanism need further study.

5. Conclusion

This study provided evidence that ethanol extract of *A. argyi* could inhibit the growth and the biofilm formation of *S. aureus*, and could protect mice from death induced by *S. aureus* infection. As a traditional Chinese medicine, *A. argyi* could be a potential medicine for therapy in *S. aureus* infection.

Acknowledgements

This work was supported by the Natural Science Foundation of Anhui Province (1308085MH134). The authors would like to thank the Clinical Laboratory of the First Affiliated Hospital of Anhui Medical University for providing the bacterial strains.

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