Antimicrobial Activity Study of Chinese Medicine Madeng'ai

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Research

Keywords: Madeng'ai, antimicrobial, MRSA, PAE, E. coli

Posted Date: February 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-181332/v1

Abstract

Background Due to the overuse of antibiotics, many multidrug-resistant bacteria have emerged, which brings huge challenges to the clinical treatment of bacterial infections. New products for anti-infection are necessary.

Methods *Madeng'ai* powder was added with Milli-Q water or LB culture and autoclaved to prepare medicine suspension at different concentration. Bacteria were cultured in LB with different concentration of *Madeng'ai.* and swab on LB agar plates to get minimal inhibitory concentration (MIC) of *Madeng'ai.* Mice back was cut to make wound and MRSA/PAE suspension was injected in the wound area. Then swab with *Madeng'ai* extracts. Bacteria growth of infected secretions was checked on LB agar, and Hematoxylin and eosin (H&E) staining was performed for Histological analysis of skin tissues infected with bacteria after *Madeng'ai* and PBS (control) treatment.

Results *Madeng'ai* could widely inhibit *E.faecalis*, *Pseudomonas aeruginosa* (PAE), *Klebsiella pneumoniae* (*K.pneumoniae*) and *Acinetobacter baumannii* (*A.baumannii*) at concentration of 4.0 mg/ml. The mice model also showed that *Madeng'ai* had imposed restrictions on MRSA and PAE growth *in vivo*.

Conclusion Here, we report that a new Chinese medicine *Madeng'ai* has antimicrobial activity functions *in vitro* and *in vivo.* These data briefly showed that *Madeng'ai* functioned on antimicrobial and provided a new consideration for an antibiosis product.

Introduction

Since the antibiotic era, when Alexander Fleming discovered the first antibiotic, penicillin, in 1928, antibiotics have been commonly used for infectious diseases, benefiting both animal and human health. Although the variety and generation of antibiotics were developed rapidly in the recent century, the overuse and misuse of antibiotics have boosted the prevalence and severity of antimicrobial resistance (AMR), which is recognized as an international public health threat[1, 2]. In Norway, Iceland, and European Union, the European Centre estimated that 25,000 deaths were caused by AMR annually from 2009, while in the USA, AMR caused 99,000 deaths each year[3, 4]. AMR's phenomenon has increased dramatically, and its severity has been aware of adequately in recent decades. In the USA, the AMR's concern is reflected in the Center for Disease Control (CDC) published a report "Antibiotics Resistance Threats in the United States, 2013". In the UK, the National Risk Assessment (NRA) released a report on the number of infectious cases with AMR and was estimated to increase significantly. In China, the National Health and Family Planning Commission reported the "National Action Plan for Containing Antibacterial Resistance (2016-2020)" in 2016. AMR is always with uncontrolled infection, which increases the risk of sepsis, septic shock, microbial toxic syndrome, and even mortality. In 2017, 48.9 million worldwide sepsis cases were estimated, while 11 million deaths complicated with sepsis were reported[5]. In Japan, a prospective nationwide cohort study of sepsis was conducted by Yutaka Umemura and colleagues from 2016 to 2017. In their study, they found that the most common pathogen of sepsis was *Escherichia coli* (*E. coli*),

the most frequent Gram-positive bacteria were *Staphylococcus aureus* (*S.aureus*), the fatal one was Methicillin-resistant *Staphylococcus aureus* (MRSA). Besides, Gram-positive bacterias were slightly more common in sepsis compared to Gram-negative bacterias[6]. Similarly, Edward Goldstein and his colleagues reported that hospitalization rates with sepsis and related mortality increased remarkably in the recent two decades. *E.coli* corresponded the most substantial role in associating with septicemia hospitalization rate in elderly adults (>50 y), as well as the rate of sepsis mortality in adults (18-84y)[7]. In China, Xinchuan Chen and colleagues kept their eyes on the distribution of sepsis since they were concerned that sepsis had played a common and essential role in increasing the risk of mortality. They found that Gram-positive bacteria are similarly prevalent to Gram-negative bacteria in sepsis among adults. Gram-positive bacteria-related sepsis was more common for neonates than Gram-negative bacteria[8]. Consequently, it is essential to take action against AMR, such as limiting and monitoring antimicrobial agents' use and developing new antimicrobial agents and alternatives[2, 9].

Honey, the natural product, was most commonly used as a flavoring agent. In the medical field, honey has been topically used for wound and burn treatment initially for millennia. Manuka honey was reported to inhibit a broad spectrum of microorganisms and exhibit antimicrobial activity with a high efficacy[9]. *Artemisia argyi*, also called Chinese mugwort, has been widely used as a traditional Chinese herbal medicine for millennia. Xiao Guan and colleagues published their findings that simultaneous distillation extracted *Artemisia argyi Levl.et Vant* essential oil exhibited a high efficacy in antimicrobial activity, such as for *Listeria monocytogene, Escherichia coli, Proteus Vulgaris, Salmonella enteritidis and Aspergillus niger,* which is especially significant for Gram-positive bacterias[10]. *Potentilla*, another traditional Chinese herb, a member of the family Rosaceae, has been accepted by *Plants of the World Online* and founded it natively ranged from Subarctic & Temp. to Tropical Mountains. Shan-Shan Wang and colleagues found that leaf extracts of three *Potentilla* species (*Potentilla fruticosa, Potentilla glabra, and Potentilla parvifolia*) exhibited a significant inhibitory effect on microbial pathogens. They also reported that *Potentilla fruticosa* showed the most potent inhibition against Gram-positive bacteria and *Pseudomonas aeruginosa*, while *Potentilla parvifolia* exhibited a broad spectrum of antifungal and antibacterial activities[11]. Consequently, in recent decades, it is widely accepted that some herbs and natural plants have great potential in utilizing as alternatives against microorganisms. The species of *Potenllia freyniana Bornm* also accepted by the *Plant of the World Online* and the *Plant List,* a member of the genus *Potenllia* and the family *Rosaceae*, predominantly distributed from Russian Far East to China and Tem. E Asia. *Madeng'ai*, a variant of *Potenllia freyniana Bornm.* (Figure1), has been widely used in a branch of traditional Chinese medicine, called Ethnic *Dong* minority's traditional medicine, mainly distributed in southern China, like Hunan, Guizhou, and Guangxi provinces. In the Ethnic *Dong* minority, *Madeng'ai* has been used to inhibit infectious diseases with a long history in folk medicine, especially for traumatic and wounded skin infection, which inspires us that *Madeng'ai* may contribute to antibiotic-resistant infection. However, its function in anti-infection has not been studied. Here, we reported that *Madeng'ai* had antimicrobial activity *in vitro* and *in vivo*. It could be widely used to anti-*Staphylococcus aureus,E.faecalis*, PAE, *K.pneumoniae, and A.baumannii*.

Method And Materials

Plant material

Madeng'ai samples were collected from Grassland above 800 meters in Tongdao County, Huaihua City, Hunan Province, and identified by the Hunan Provincial Key Laboratory of Dong Medicine, Hunan University of Medicine. Collected plant materials were powdered and stored at 4℃ until further analysis.

Experimental animals

Preparation of the extract (medicine suspension)

Weigh 10g powder of the medicine (*Madeng'ai)*, add 50ml autoclaved Milli-Q water, then autoclave it and get the pasty medicine suspension (0.2 g/ml).

Microorganisms

Six bacterial strains were provided by the First Affiliated Hospital of Chongqing Medical University, China. The strains used are *Staphylococcus aureus* (*S. aureus*, ATCC25923), *Escherichia coli* (*E. coli*, ATCC259220), *Enterococcus faecalis* (*E.faecalis*, ATCC29212), *Pseudomonas aeruginosa* (PAE, ATCC2785), as well as *Klebsiella pneumoniae* (*K.pneumoniae*) and *Acinetobacter baumannii* (*A.baumannii*). The latter two were isolated from the patient's sputum, and their drug susceptibility tests (Minimum inhibitory concentration, MIC) were non-multiple antimicrobial resistant.

Minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of *Madeng'ai* extracts for antimicrobial testing was determined by the LB agar plate. The solvent without extracts was served as the negative control, and the others in different extract concentrations were the experimental groups. Inhibition of organism growth in the plates containing test crude extracts was judged by comparing growth in blank control plates. The MIC values were determined as the lowest concentration of extracts inhibiting visible growth of each organism on the agar plate. All the samples were tested in triplicate.

In vitro antimicrobial activity

Preparation of test solution

Madeng'ai powder 0mg, 100mg, 200mg, 300mg, 400mg, and 500mg were added into six flasks containing 100ml LB culture respectively, and high temperature autoclaved to prepare extract medicine suspension concentration at 0 mg/ml, 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, and 5 mg/ml. Then, 100 μl aliquots of culture containing MRSA or *E.coli* were added into six 100ml flasks, respectively, and shaking cultured at 37℃, 220 round/min for 2-3 hours until OD_{600nm} = 0.5. Finally, 100 μl aliquots from six kinds of culture containing MRSA or *E.coli* at different medicine suspension concentration (0 mg/ml, 1mg/ml,

2mg/ml, 3mg/ml, 4mg/ml, and 5 mg/ml) were pick up and spread on normal LB agar to culture overnight to get the optimum concentration.

Microorganisms for animals

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (PAE) were cultured in LB broth at 37℃, 220 round/min for 2-3 hours until OD_{600nm} = 0.5.

Animal model

All animal experiments were performed under the experimental animal use guidelines of the National Institutes of Health. All procedures for the mouse experiments were approved by the Ethics Committee of Animal Experiments of Chongqing Medical University. All mice were housed in laminar flow cabinets under specific pathogen-free conditions at room temperature with a 12 h light/dark cycle, with food and water available ad libitum in the Experimental Animal Centre, Chongqing Medical University. 12 female BABL/c mice (5-6 weeks old) were randomly divided into 4 groups (MRSA-medicine group, MRSA-PBS group, PAE-medicine group, PAE-PBS group). The back of the mice was unhaired and cut a 0.5cm diameter wound on the mice's surface with a sterilized surgical scissor, then subcutaneously 100μL MRSA/PAE suspension was injected in the wound area. After 48h, the wound surface was swab with extracts (*Madeng'ai*, medicine suspension) by the cotton swab twice a day for the MRSA/PAE-medicine groups; similarly, PBS was swab as control twice a day for the MRSA/PAE-PBS groups.

In vivo antimicrobial activity

Changes of mice weight and wound recovery condition

Mice body weights and wound recovery conditions were recorded daily for 10 days after swabbing the extracts (*Madeng'ai*, medicine suspension) and control (PBS) s treatment.

Bacteria growth condition and histological analysis after treatment

After 10-day treatment with extracts (*Madeng'ai,* medicine suspension) and PBS, 75% ethanol was used to sterilize wound surfaces (recovered or unrecovered) of the mice. The wound surfaces were then cut down with the sterilized surgical scissor, swabbed on the LB agar plates for bacteria culture, Then, wound surfaces were cut into 4 μm tissue slices and subjected to hematoxylin and eosin (H&E) staining for histological analysis.

Statistical analysis

All experimental data are expressed as the means and standard error of the mean (SEM).

Results

Minimum inhibitory concentration (MIC)

Antimicrobial activity results of *Medeng'ai* extracts in different MIC values for common Gram-positive and Gram-negative bacteria are shown in Figure 2. The extract (*Madeng'ai*, medicine suspension) exhibited macroscopic inhibitory activity from 2.0 mg/ml against Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) as almost no macroscopic *S. aureus* colony could be observed. Since 1.0 mg/ml of the extract became to affect *S. aureus* grown on the LB agar plate as less *S. aureus* colonies were observed compared to those without extracts. Similarly, the extracts inhibited Gram-negative bacteria *Escherichia coli* (*E. coli*) on the LB agar plate. The best inhibitory activity was detected against *E. coli* with 4.0 mg/ml of the extract as no macroscopic *E. coli* colony could be observed, though 1.0 mg/ml of the extract became to affect *E. coli*, 2.0 mg/ml and 3.0 mg/ml almost inhibited *E. coli* grown in the on the LB agar plate as less *E. coli* colonies were observed than those without extracts.

To further explore the antimicrobial spectrum of *Madeng'ai* extracts, the antimicrobial activity of them for more bacteria in the optimum concentration were tested, and results are shown in Figure 3. In the concentration of 4.0 mg/ml, the extracts showed macroscopic inhibitory activity against Gram-positive bacteria *Enterococcus faecalis* (*E.faecalis*) and Gram-negative *Pseudomonas aeruginosa* (PAE). Another two Gram-negative bacterias, *Klebsiella pneumoniae* (*K.pneumoniae*) and *Acinetobacter baumannii* (*A.baumannii*), isolated from patient's sputum, were detected inhibited obviously by 4.0 mg/ml of the extracts as no bacterial colonies could be observed on the LB agar plate.

Antimicrobial activity function *in vivo*

In the MRSA-medicine and MRSA-PBS groups, wound recovery conditions are shown in Figure 4. On Day 0, before treatment, infected wound areas on BALB/c mice could be observed obviously in both the MRSA-medicine group and MRSA-PBS group. On Day 6, after treatment, infected wound and infiltration areas were smaller than those on Day 2 in the MRSA-medicine group, the margin of the infected wound was more distinct and more regular than that on Day 2, and secretion of the infected wound was less than that on the Day 2. In contrast, control mice (MRSA-PBS group) had larger infected wound and infiltration areas on Day 6 than those on Day 2, as well as the margin of the infected wound on Day 2. It was vaguer, less regular, and less secretion on Day 2 than it on Day 6. On Day 10, the infected wound in the MRSA-medicine group recovered obviously as no redness and swelling, no macroscopic wound surface, compared to that in the MRSA-PBS group, which was apparent infected open wound with redness and swelling.

In the PAE-medicine and PAE-PBS groups, wound recovery conditions are shown in Figure 4. (A). Similarly, the infected wound recovered better in the PAE-medicine group than those in the PAE-PBS group. On Day 0 (before treatment), infected wound areas on BALB/c mice could be observed obviously in both the PAE-medicine group and PAE-PBS group. On Day 6, after treatment, in the PAE-medicine group, the infected wound and infiltration areas were smaller than on Day 2. The infected wound margin was more distinct and more regular, secretion of the infected wound was also less than those on Day 2. On Day 10, the infected wound in the PAE-medicine group recovered obviously like that in the MRSA-medicine group, with

no redness and swelling, neither nor macroscopic wound surface. However, an infected wound was still observed in the PAE-PBS group as it was red, swollen, and open wounded on Day 10 after treatment.

Bacteria growth condition and histological analysis after treatment

After completed treatment, MRSA and PAE growth conditions of infected secretions from wound surfaces (recovered or unrecovered) are shown in Figure 4. (B). and Figure 5. *(B*), respectively. After treating extracts (*Madeng'ai*), bacterial residues of MRSA on the LB agar plate were almost negligible as no MRSA colony could be observed, while a large number of MRSA colonies were observed with PBS treatment in the control group. Similarly, the bacteria residue of PAE was almost none after extract treatment as no PAE colony could be observed on the LB agar plate, and a large amount of PAE colonies were still observed on the LB agar plate with PBS treatment in the control group.

Histological analysis (H&E staining) of wound surface (recovered or unrecovered) slices after completed treatment are shown in Figure 4. (C) and Figure 5. (C). A large number of inflammatory cells (mark with a red circle) which are in the bluish violet color were observed on the upper pictures of both Figure 4. (C) and Figure 5. (C), indicating that MRSA/PAE-infected wound after completed PBS treatment remained infection. However, on the bottom pictures of both Figure 4. (C) and Figure 5. (C), the number of inflammatory cells was reduced obviously after extracts (*Maadeng'ai*, medicine suspension) treatments in both the MRSA-medicine group and PAE-medicine group. Besides, the epidermis was more intact after treatment than in the control group with PBS treatment, which indicated that the infected wound surface had recovered after extracts (*Madeng'ai*, medicine suspension) treatment.

Discussion

In our study, the most common infectious bacteria, *Staphylococcus aureus* (*S. aureus*), was inhibited by the extract (*Madeng'ai,* medicine suspension) in 2.0mg/ml on the LB agar plate, while another the most common infectious bacteria *E. coli* was detected the best inhibitory activity by the extract in 4.0 mg/ml on the LB agar plate. It gives us an idea that the extract's antimicrobial activity for a broad spectrum of bacteria, including Gram-positive and Gram-negative bacteria, could be expressed as the best inhibitory result in 4 mg/ml. Consequently, we supposed that 4 mg/ml of the extract could also inhibit further bacteria growth. As expected, other common infectious bacteria *E.faecalis*, PAE, *K.pneumoniae*, and *A.baumannii* on the LB agar plates were inhibited by 4.0 mg/ml of the extracts (*Madeng'ai, medicine* suspension). *In vivo*, the extract of medicine suspension (*Madeng'ai*) exhibited similar antimicrobial activity in mice as that *in vitro*. The mice, either topical infected by Methicillin-resistant MRSA or PAE, wound surfaces were macroscopically recovered better with medicine (*Madeng'ai*) treatment comparing to those treated with PBS as control. Moreover, MRSA's and PAE's bacterial residues, which were cultured from the infected wound surface's secretions, were almost negligible on the LB agar plates after medicine (*Madeng'ai*) treatment. Besides, histological analysis (H&E staining), exhibiting infected wound surfaces had recovered after medicine (*Madeng'ai*) treatment.

Up to now, a large number of drugs have been founded to exhibit their antimicrobial activities and prescribed widely in the clinics. Anne H. Norris and colleagues recommended that the choices of antimicrobial depended more on the outpatient parenteral antimicrobial therapy model than on the pharmacokinetic properties of the drug[12], and antibiotics such as only vancomycin, cefazolin, or the aminoglycosides may be limited to select for patients who received parenteral antimicrobials during dialysis sessions[13]. Shanqing Li and colleagues illustrated that the basic principles about deciding antimicrobial prophylaxis should be based on any contamination in the surgical field. For instance, antibiotics should prevent postoperative surgical site infection (SSI) as well when they selected to against *S. aureus*, drugs should be selected before a colon and rectal surgery when they were selected to inhibit *E.coli* [14]. Empirical therapy for a bacterial infection has been widely accepted since it is essential and effective. For instance, vancomycin and antistaphylococcal penicillin are used empirically to inhibit *Staphylococcus aureus* bacteremia; vancomycin is the first choice for MRSA treatment[15-17]. Fosfomycin-trometamol and nitrofurantoin have been recommended as first-line antibiotics for frequently uncomplicated cystitis; fluoroquinolones are the oral first-line treatment for uncomplicated pyelonephritis as *E.coli* is the leading uropathogen of uncomplicated urinary tract infections[18-20].

With the development of antibiotics, researchers and clinicians have found that antimicrobial resistance is an important factor in impeding antimicrobial activity. Thus, they also put the eyes on exploring the mechanisms of antimicrobial resistance. Antimicrobial resistance could happen when the target cell envelope's permeability is limited or reduced specifically by bacteria to a given antibiotic, such as *Pseudomonas aeruginosa*; similarly, when chemical affinity was lost between antimicrobial and its target, such as streptococci[21]. The antimicrobial ability could be degraded by the bacterium, such as by β-lactamase production, as expanded spectrum β-lactamase could bring to the emergence of new and nonresponsive bacterial clones[22]. In addition, some other mechanisms of antimicrobial resistance have been reported. For instance, antimicrobials could be pumped out from bacterial cells by efflux systems[23], and the extracellular polymer substances produced by biofilms could resist antimicrobial drugs to interrupt molecular diffusion through electrostatic and steric interactions[24]. Thus, researchers discovered more antimicrobial agents to alternate traditional antibiotics when mechanisms of antimicrobial resistance were more explicit.

Although the antimicrobial mechanism of traditional Chinese medicine *Madeng'ai* in our study is still unclear, many studies have reported phenolic compounds of traditional Chinese medicine *Potentilla* species could be the critical factor in their antimicrobial effects[11, 25]. It inspires us an idea about future work direction in exploring the antimicrobial mechanism of traditional Chinese medicine *Madeng'ai*, a variety of *Potenllia freyniana Bornm* that could be considered relative to phenolic compounds as well. Furthermore, *Potentilla* species display anti-inflammatory and vasoconstrictive effects in treating topical inflammatory skin disorders[26-28] and alleviating diabetes mellitus function[29, 30]. Consequently, traditional Chinese medicine *Madeng'ai* has great potential not only in antimicrobial effect but also in other fields, which is worthwhile to explore more in our future work.

Conclusion

Here, we report that a new Chinese medicine, *Madeng'ai* has antimicrobial function *in vitro* and *in vivo.* It would be as a potential anti-inflammatory drug in the future.

Declarations

Ethics approval and consent to participate

All animal experiments were performed under the experimental animal use guidelines of the National Institutes of Health. All procedures for the mouse experiments were approved by the Ethics Committee of Animal Experiments of Chongqing Medical University.

Consent to publish

All authors have agreed to publish this manuscript.

Competing interests

No competing interest was reported by the authors of this paper.

Authors' Contributions

Conceptualization –Hua Tang, Zaiqi Zhang, **Data curation –**Zhenrong Tang, Yannan Zhao, **Formal analysis –**Dan Wang, **Funding acquisition–** Hua Tang, **Investigation –**Yannan Zhao, Huan Yue,**Methodology –**Jiangling Wu, Dongmei Xiong, **Project administration –** Hua Tang, **Resources –** Chunsheng Liu, **Supervision –**Chunsheng Liu, Hua Tang, **Visualization –**Yannan Zhao, Hua Tang, **Writing – original draft –**Zhenrong Tang, **Writing – review & editing –**Chunsheng Liu, HuaTang.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Funding and Acknowledgement

This study was supported by the research plan of the Hunan Provincial Key Laboratory of Dong Medicine (2015TP1020-04, 2017CT5025). Thanks to Dr. Yuyi Ren (Institute of Ophthalmology, Faculty of Brain Sciences, University College London, UK) for her kind manuscript revising.

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Figures

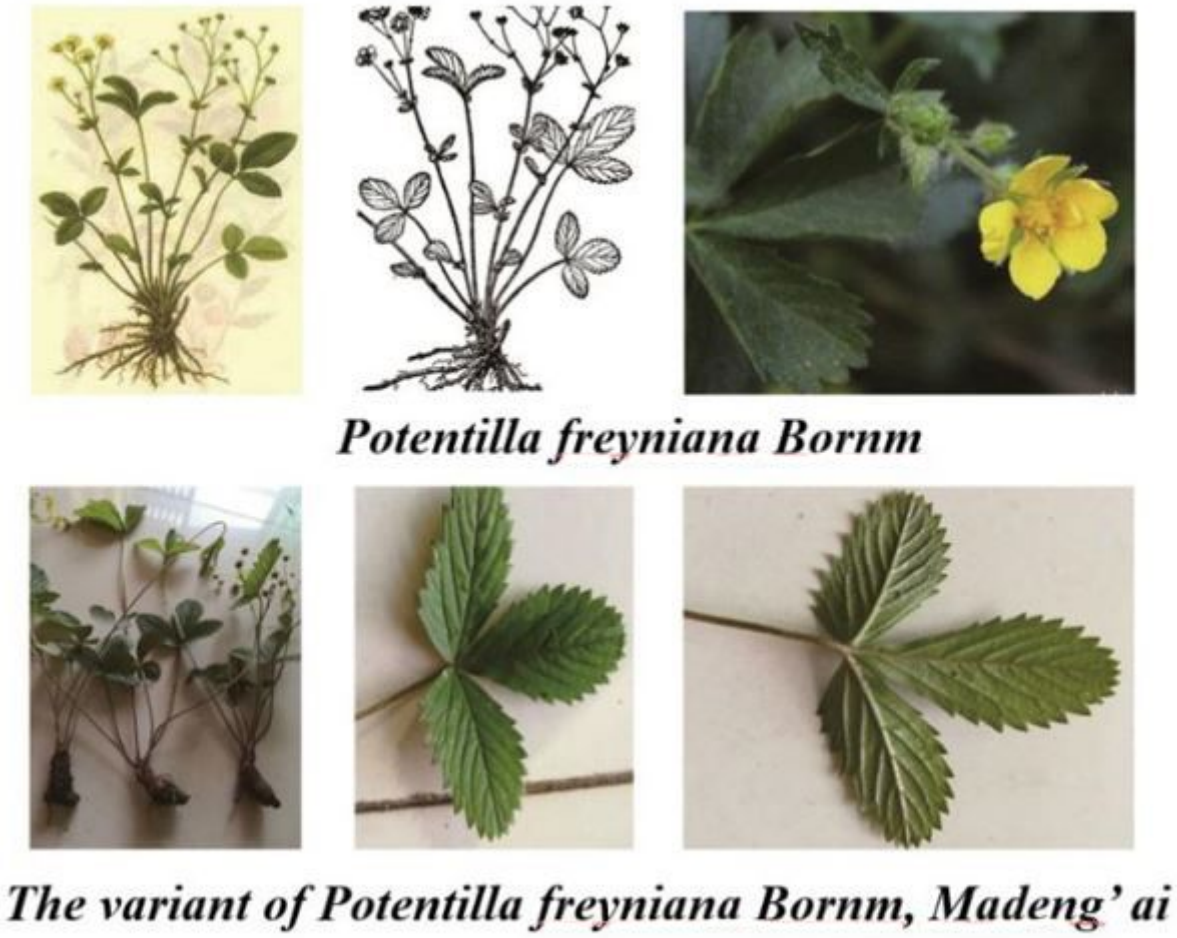

Figure 1

The appearance of Madeng'ai and Potentilla freyniana Bornm. The upper three pictures are the appearances of Potentilla freyniana Bornm. The bottom three pictures are the appearances of Madeng'ai (a variant of Potentilla freyniana Bornm.).

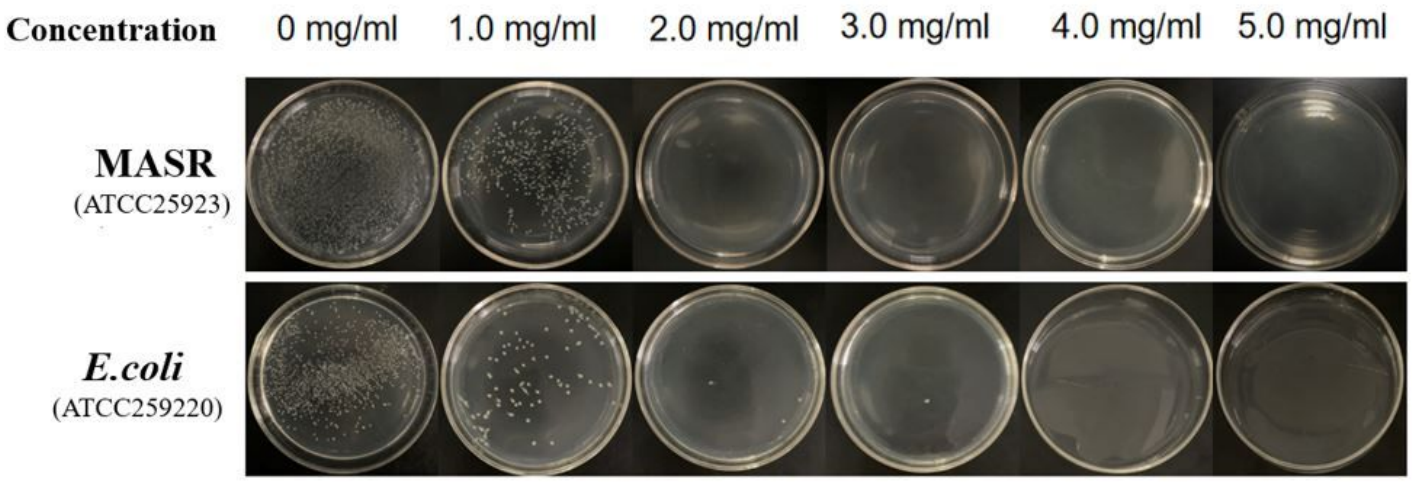

Figure 2

MIC values of traditional Chinese medicine Madeng'ai for inhibiting S.aureus and E.coli.

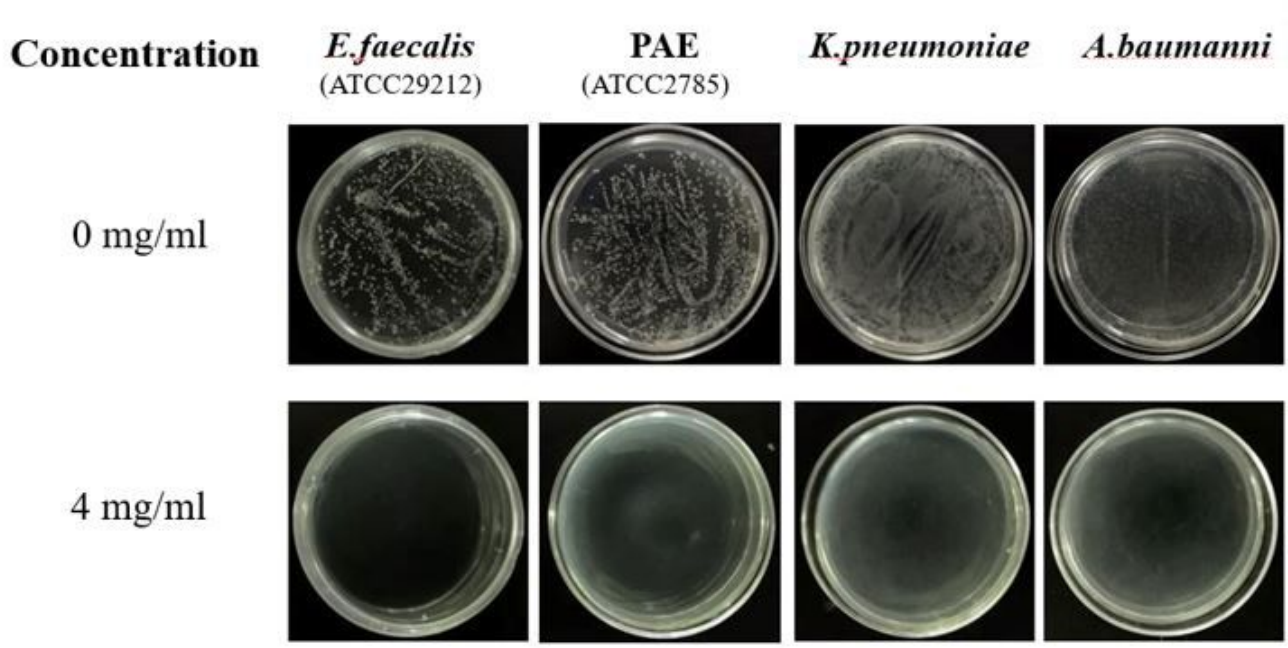

Figure 3

The inhibitory effect of Madeng'ai on four bacteria colonies.

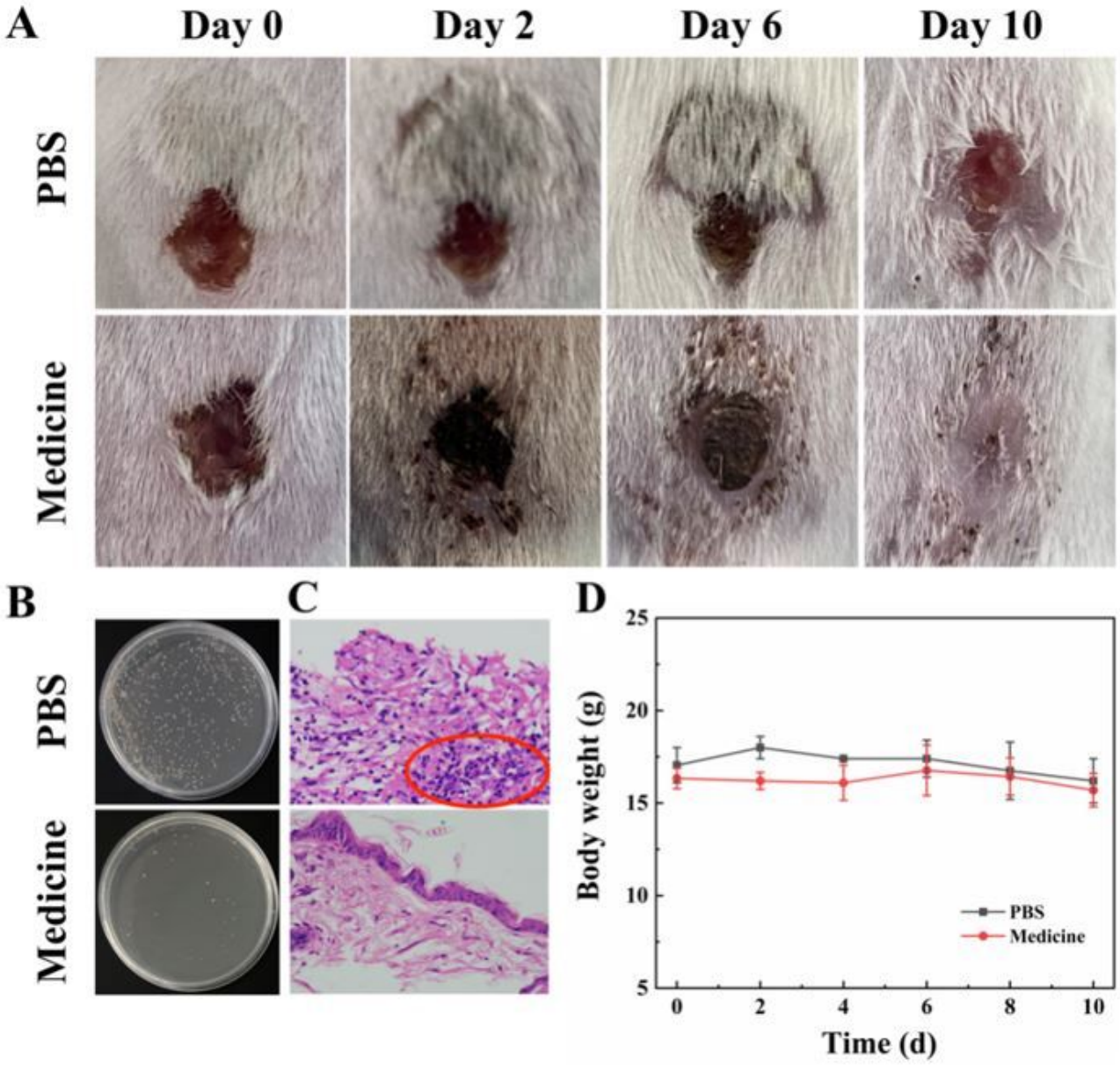

Figure 4

Bacteria growth and histological analysis after Madeng'ai treatment for MRSA. (A) Infected wound recovery on BALB/c infected with MRSA, treated with Madeng'ai and PBS (control). (B) Bacteria growth of infected secretions on LB agar after treatments with Madeng'ai and PBS (control). (C)Histological analysis of skin tissues infected with bacteria after Madeng'ai and PBS (control) treatment by hematoxylin and eosin (H&E) staining. (D) Changes in body weight of BALB/c during treatments.

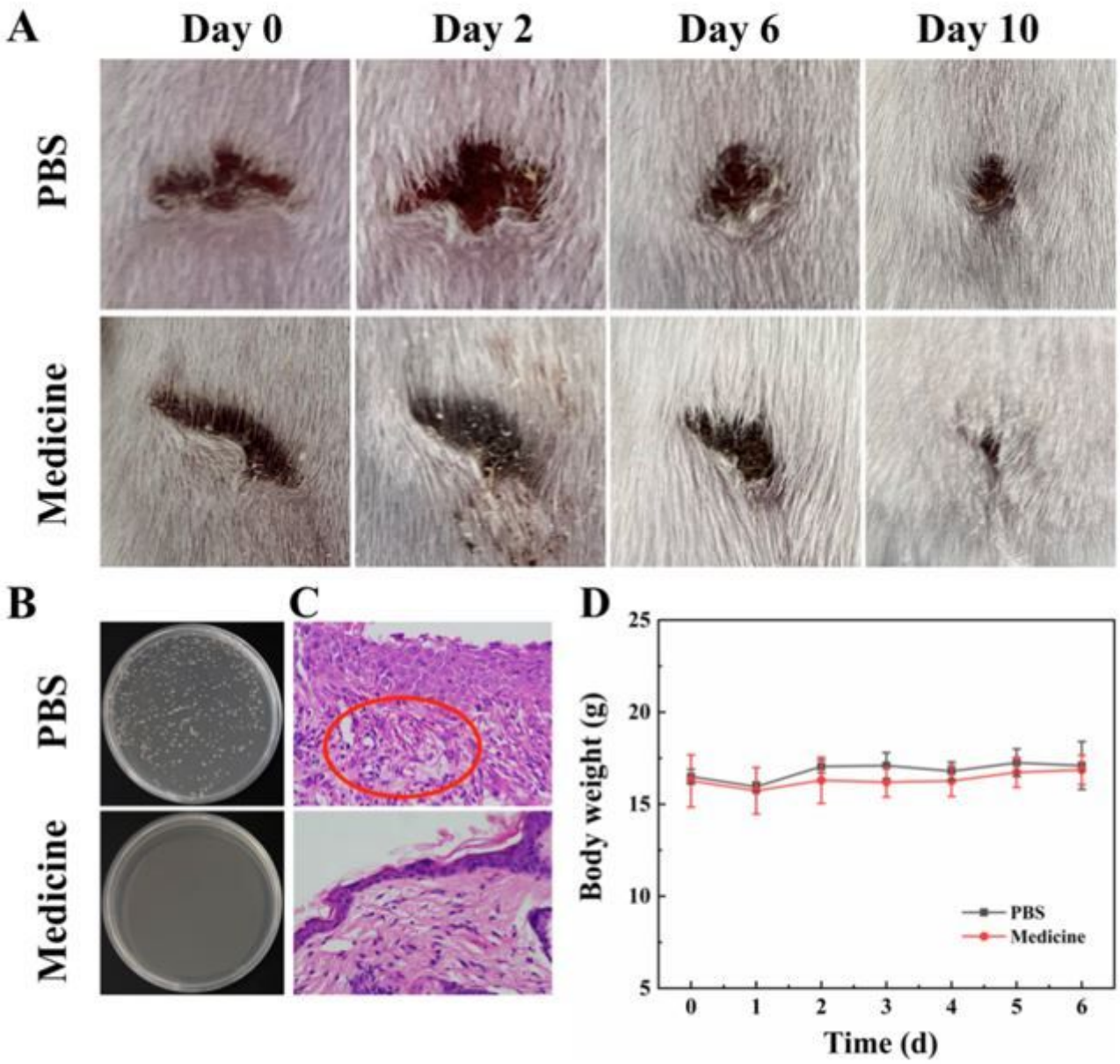

Figure 5

Bacteria growth and histological analysis after traditional Chinese medicine Madeng'ai treatment for PAE. (A) Infected wound recovery on BALB/c infected with PAE, treated with Madeng'ai and PBS (control). (B) Bacteria growth of infected secretions on LB agar after treatments with Madeng'ai and PBS (control). (C)Histological analysis of skin tissues infected with bacteria after Madeng'ai and PBS (control) treatment by hematoxylin and eosin (H&E) staining. (D) Changes in body weight of BALB/c during treatments.