EFFICACY OF *AMOMUM TSAO-KO* CREVOST & LEMARIÉ ESSENTIAL OIL IN INTRAGASTRIC TREATMENT OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* INFECTED MICE

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Abstract. Efficacy of *Amomum tsao-ko* Crevost & Lemarié (also known *Lanxangia tsaoko* (Crevost & Lemarié) MF Newman & Škorničk) as essential oil against methicillin-resistant *Staphylococcus aureus* (MRSA) was evaluated in an *in vivo* mouse model. Minimal lethal dose (MLD) of MRSA on Day 7 following intraperitoneal injection was 6.0×10^{10} CFU/kg body weight. Preventive efficacy was evaluated by intragastric application of *A. tsao-ko* essential oil for three days, then an administration of MRSA MLD to mice (n = 10), demonstrating a 50% effective dose (ED_{50}) on Day 7 post-last treatment of 0.42 g/kg body weight/day; while therapeutic efficacy was evaluated by administration of MRSA MLD then intragastric treatment with *A. tsao-ko* essential oil for three consecutive days before determining ED_{50} on Day 7 post-last treatment, with a value of 0.73 g/kg body weight/day. *A. tsao-ko* essential oil ameliorated inflammatory response caused by MRSA infection through regulating serum levels of inflammatory cytokines IL-1β (partially), IL-6, and TNF-α, and histopathological examination demonstrated protection against tissue damages to kidney, liver and lung (partially). These findings suggest *A. tsao-ko* essential oil as a potential treatment of MRSA infection in animals and possibly humans.

Keywords: *Amomum tsao-ko*, ED_{50}, essential oil, histopathological change, inflammation, methicillin-resistant *Staphylococcus aureus*

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first detected in 1961 following introduction of methicillin in 1959 (Jevons, 1961). MRSA, known as "super bacterium", is one of the most important drug-resistant pathogens for

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nosocomial and community-acquired infections. MRSA causes soft tissue infection, pneumonia, endocarditis and sepsis, and is responsible for 25-50% of the world's nosocomial *S. aureus* infection, which together with hepatitis B virus and human immunodeficiency virus constitutes the three major infectious pathogens (Frazee *et al*, 2005; Malani, 2014; Xu-hong *et al*, 2014; Bosch *et al*, 2015).

At present, vancomycin is the main drug for the treatment of MRSA infection, but as vancomycin-susceptible bacteria have continuously declined and the drug has adverse side effects (Chen, 2013; Álvarez *et al*, 2016), it is urgent to seek new and safer alternative agents for treating MRSA infection. Compared with antibiotics, Chinese traditional medicine has many advantages, such as relatively low toxicity, low drug residue, non-specific antibacterial effect with less ability to develop drug resistance, and ability to regulate and improve the body's immune capacity (Chen *et al*, 2015; Tan *et al*, 2015; Wang *et al*, 2017). Thus, Chinese traditional medicine provides a great potential source from which to develop antibacterial drugs.

Amomum tsao-ko Crevost & Lemarié (also known *Lanxangia tsaoko* (Crevost & Lemarié) MF Newman & Škorničk) is a perennial herb of the ginger family widely grown in southwestern China (Delin and Larsen, 2000). As a food flavoring, it is used as a condiment for cakes, foods and hotpots. *A. tsao-ko* is commonly used to treat a variety of gastrointestinal diseases in China (Min *et al*, 2016; Rahman *et al*, 2017) and an antibacterial active component of *A. tsao-ko* is its essential oil (Cui *et al*, 2017). *A. tsao-ko* essential oil has an effective anti-MRSA activity *in vitro* (Hang *et al*, 2017). As there is no study on the effect of *A. tsao-ko* essential oil on anti-MRSA infection *in vivo*, here the *in vivo* anti-MRSA infection efficacy of *A. tsao-ko* essential oil was evaluated to provide a foundation for subsequent research on a comprehensive development and utilization of *A. tsao-ko* essential oil as a bactericidal agent in humans and animals.

MATERIALS AND METHODS

Bacteria strain and materials

MRSA ATCC43300 was deposited with Sichuan Provincial Experimental Teaching Demonstration Center of Medical Laboratory, Chengdu Medical College, Sichuan, China. *A. tsao-ko* Crevost & Lemarié was provided by Beijing Tong Ren Tang (Beijing, China) (batch no. MKBQ1662V), vancomycin from Bioengineering (Shanghai) Co, Ltd (Shanghai, China) (30 mg/ml with physiological saline), Tween-80 from Sinopharm Chemical Reagent Co, Ltd, (Shanghai, China) (batch number 20150429), porcine stomach mucin from Sigma (St Louis, MO) (batch no. SLBP2671V), nutritional agar (NA) from Beijing AOB Star Biotechnology Co, Ltd (Beijing, China) (batch no. 20150810), and ELISA kit (for murine IL-1β, IL-6 and TNF-α) from Elabscience (Wuhan, China).

Animals

Kunming mice, SPF grade (n = 180, equal number of females and males of 20 ± 2 g) provided by the Chengdu Institute of Biological Products Co, Ltd (Chengdu, China) (animal production license no. SCXK (Chuan) 2016-08) were kept under a good laboratory animal care. Research protocols were conducted in compliance with the Animal Ethics Review Committee of Chengdu Medical College (approval no. 20170802).

Extraction of *A. tsao-ko* essential oil

A. tsao-ko essential oil was extracted by a steam distillation method (Xu-hong *et al*, 2014). In brief, *A. tsao-ko* fruits were broken open and 40 g of kernel were soaked in 2 liters of distilled water for two hours, followed by steam distillation and essential oil was collected after four hours, density measured (p = 929 g/l) and stored in brown bottle at 4°C until used.

Minimum lethal dose (MLD) of MRSA in mice

Mice were randomly divided into seven groups (10 mice per group), 6 experimental groups and one control group, after they adapted to the environment. MRSA was grown in TSA, harvested at logarithmic growth phase and suspended in physiological saline**.** Each bacterial suspension in 5% (w/v) mucin was intraperitoneally injected into six groups of mice. Control group was treated with vehicle. The minimum bacterial concentration that caused death of all mice on Day 7 post-injection is defined as MRSA minimum lethal dose (MLD).

Efficacy of *A. tsao-ko* essential oil against MRSA infection

Efficacy of *A. tsao-ko* essential oil against MRSA infection was determined by measuring prevention and treatment effects. For prevention efficacy, mice were divided into nine groups (five females and five males per group) comprising five test groups, a blank control group (given saline by intragastric administration), a model control group (given saline by intragastric administration), a solvent control group (given 1% Tween-80 vehicle by intragastric administration), and a positive drug control (given vancomycin (0.30g/kg) by intragastric administration). Each of five test groups was given by intragastric administration 0.17, 0.33, 0.47, 0.65, and 0.93 g of *A. tsao-ko* essential/ml of 1% Tween-80, respectively once a day for three days. Except for the blank control group, mice were challenged with MRSA MLD subsequent intragastric treatment on Day 3. On Day 10 following MRSA administration, number of surviving mice per group was recorded (Fig 1). For therapeutic efficacy of *A. tsao-ko* essential oil, mice were divided into the nine groups as described above. All groups were first given MRSA MLD by intragastric administration, then treated with *A. tsao-ko* essential oil as described above. On Day 7 following the last treatment, number of surviving mice per group was recorded (Fig 1).

Determination of *A. tsao-ko* essential oil on mice cytokines levels

On Day 7 following last administration of *A. tsao-ko* essential oil in the therapeutic efficacy experiments, sera were collected from *A. tsao-ko* essential oil treated group (0.93 g/kg body weight/day), positive drug control group (0.30 g/kg body weight/day vancomycin), blank control group, solvent control group (on Day 4 as no mice survived to Day 7), and model control group mice (on Hour 12 as no mice survived beyond 24 hours) and assayed for levels of IL-1β, IL-6 and TNF-α using ELISA kit (Elabscience Biotechnology Co, Ltd, Wuhan, China).

Histopathological examination

Three surviving mice in the *A. tsao-ko* essential oil treated group were randomly selected, euthanized and liver, kidney and lung removed. Specimens were rinsed with saline and treated with 4% formaldehyde solution for 24 hours, then placed in fresh 4% formaldehyde solution prior to embedding in wax and slicing into 5 μm sections (Rotary Microtome, Leica-2016, Germany). Tissue slices were stained with hematoxylin and eosin, then

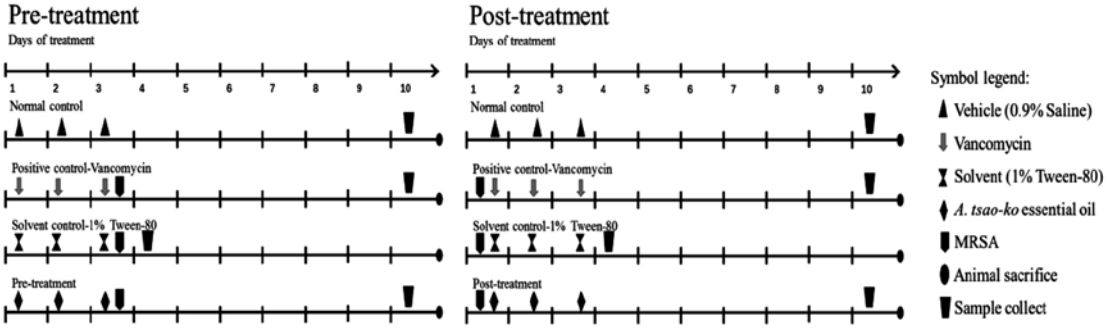

Fig 1-Workflow in determination of protective (pre-treatment) and therapeutic (post treatment) efficacies of *Amomum tsao-ko* essential oil against methicillin-resistant *Staphylococcus aureus* (MRSA) infection in mice.

Animal were sacrificed for histopathological examination of kidney, liver and lung; sample collected was blood for serum preparation and assays of cytokines

examined under a light microscope (400x magnification) (identity of sample blinded to examiner).

Statistical analysis

Fifty percent effective dose (ED_{50}) was calculated according to Karber's method (Shariff *et al*, 2009), that is to say, in the light of dose mortality response, normal distribution of data is required, and ED_{50} is calculated by formula. Statistical analysis was carried out using a one-way analysis of variance with a Statistical Package for the Social Sciences (SPSS) software version 19.0 (SPSS Inc, Chicago, IL), and p-value<0.010 or <0.050 is considered statistically significant.

RESULTS

MRSA MLD

From challenges of six different doses of MRSA, MLD was determined to be 6.0×10^{10} CFU/kg body weight (Table 1).

Preventive and therapeutic efficacies of *A. tsao-ko* essential oil against MRSA infection in mice

A. tsao-ko essential oil was administered intragastrically once a day for three days before injection of MRSA MLD and survival rate was determined on Day 7 post-MRSA infection**.** Preventive ED_{50} of *A. tsao-ko* essential oil against MRSA infection was 0.42 g/kg body weight/day, with 100% protection achieved with 0.93 g/kg body weight/day (Table 2). *A. tsao-ko* essential oil was used to control infection by intragastric administration once a day for three days following MRSA infection. Therapeutic ED_{50} of *A. tsao-ko* essential oil against MRSA infection was 0.73 g/kg body weight/day (Table 2).

Serum levels of murine cytokines in MRSA-infected controls and *A. tsao-ko* essential oil treatment

In MRSA-infected model and Tween control mouse groups, serum levels of inflammatory cytokines IL-1β, IL-6 and TNF-α determined by ELISA increased significantly compared to blank control and returned to basal levels following treatment with *A. tsao-ko* essential oil (0.93 g/kg body weight/day) and vancomycin

Table 1
Determination of minimum lethal dose of methicillin-resistant *Staphylococcus aureus* ATCC43300 in mice.

Group	N	Dose (CFU/kg)	Number of deaths	Death rate (%)
Blank	10	-	10	100
1	10	10.0×10^{10}	10	100
2	10	8.0×10^{10}	10	100
3	10	7.5×10^{10}	10	100
4	10	6.0×10^{10}	10	100
5	10	5.0×10^{10}	8	80
6	10	4.0×10^{10}	7	70

N: number of mice.

Table 2
Preventive and therapeutic effects of *Amomum tsao-ko* essential oil in mice infected with methicillin-resistant *Staphylococcus aureus* (6.0×10^{10} CFU/kg body weight).

Group	Treatment	Dose (g/kg body weight/day)	Number. of mice	Prevention survival rate (%)	Therapeutic survival rate (%)
Blank	Saline	-	10	100	100
Model	Saline	-	10	0	0
Solvent	Tween-80	0.005/0.005*	10	0	0
Positive	Vancomycin	0.30/0.30*	10	100	100
1	*A. tsao-ko* oil	0.93/1.39*	10	100	60
2	*A. tsao-ko* oil	0.65/0.96*	10	80	60
3	*A. tsao-ko* oil	0.47/0.68*	10	60	50
4	*A. tsao-ko* oil	0.33/0.48*	10	30	30
5	*A. tsao-ko* oil	0.17/0.33*	10	0	10

*Dose of prevention experiment/dose of therapeutic experiment.

(0.30 g/kg body weight/day) on Day 7 following last administration of essential oil or antimicrobial, except for IL-1β that remained 1.125/1.549 folds above blank control in both essential oil- and vancomycin-treated groups (Fig 2).

Histopathological changes in *A. tsao-ko* essential oil-treated mice surviving MRSA infection

Lung, liver, and kidney were collected from *A. tsao-ko* essential oil-treated mice surviving MRSA MLD in the prevention

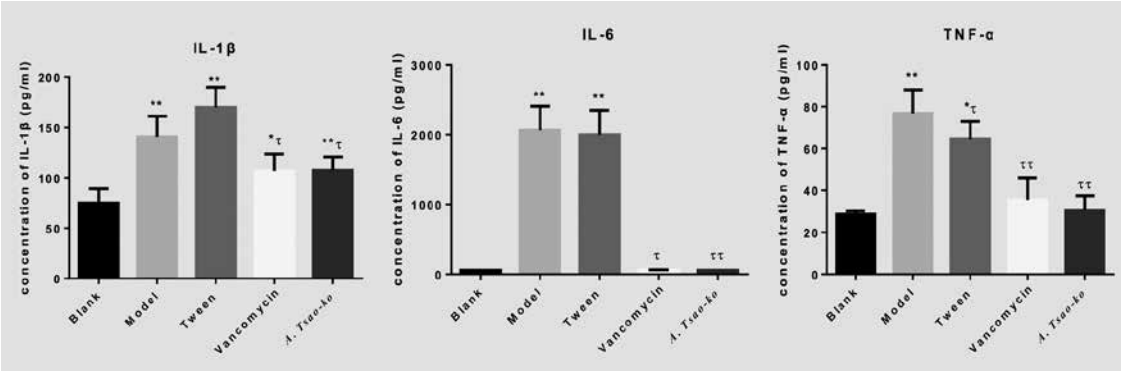

Fig 2-Serum cytokines levels in infected mice (n = 10) following treatment with *Amomum tsao-ko* essential oil or vancomycin

The experimental protocol is shown in Fig 1 (post-treatment). *A. tsao-ko*: 0.93 g/kg body weight/day; MRSA: 6.0×10^{10} CFU /kg body weight; Vancomycin: 0.30 g/kg body weight/day; Tween: 0.005g/kg body weight/day.

**p*-value <0.050; ***p*-value <0.010 compared to blank group

$^{\tau}p$-value <0.05, $^{\tau\tau}p$-value <0.010 compared to model group

experiments and histopathological features of 0.5 μm formalin-fixed hematoxylin- and eosin-stained tissue sections were observed under a light microscope (400x magnification). Compared to blank control sections, there were no obvious histopathological changes in liver (Fig 3) and kidney (Fig 4), but lung showed fused alveolar structures, which were relatively smaller in size and number compared to those observed in untreated MRSA-infected mouse (Fig 5).

DISCUSSION

Extensive use of various clinical antibacterial drugs has led to the emergence of bacterial drug resistance (Howden *et al*, 2013; Giltner *et al*, 2014; Stryjewski and Corey, 2014). Of particular concern is the presence of MRSA that has stimulated interest in the development of new anti-MRSA drugs (WHO, 2017), including ingredients from Chinese traditional medicine (Liu *et al*, 2000; Yu *et al*, 2005; Liu and Zuo, 2011). The present study shows in a mouse model *A. tsao-ko* essential oil exhibited stronger preventive than therapeutic effect against MRSA infection.

MRSA interferes with the host immune responses, leading to immune dysfunction and induction of release of a variety of inflammatory factors, such as IL-1β, IL-6 and TNF-α, which in turn directly act on vascular endothelial cells, resulting in a significant increase in permeability, thereby triggering tissue inflammatory responses and various clinical manifestations such as sepsis, shock and multiple organ failure (Kim *et al*, 2008; Veleminsky *et al*, 2008; Aliberti *et al*, 2016; Huang *et al*, 2017). Under the experimental conditions employed, *A. tsao-ko* essential oil restored serum IL-1β (partially), IL-6, and TNF-α elevated by MRSA to basal levels, and there were no obvious pathological changes in kidney, liver and lung (latter showing some residual damage to alveoli) compared to untreated MRSA-infected controls.

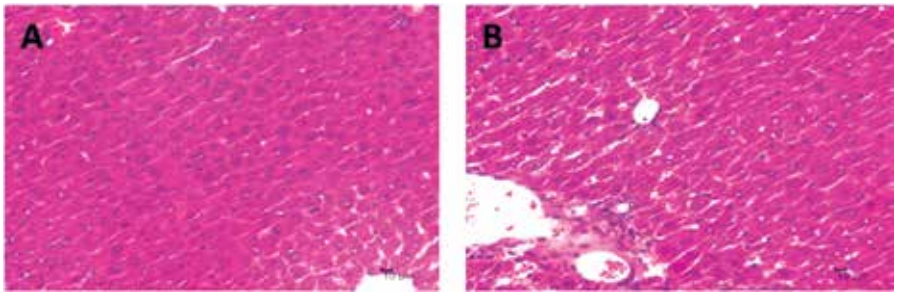

Fig 3-Pathological analysis of liver tissue in mice infected with MRSA

Compared with blank group (A), the liver tissues of model group (B) had more lymphocytes and hepatic sinus were dilated and congested (400x)

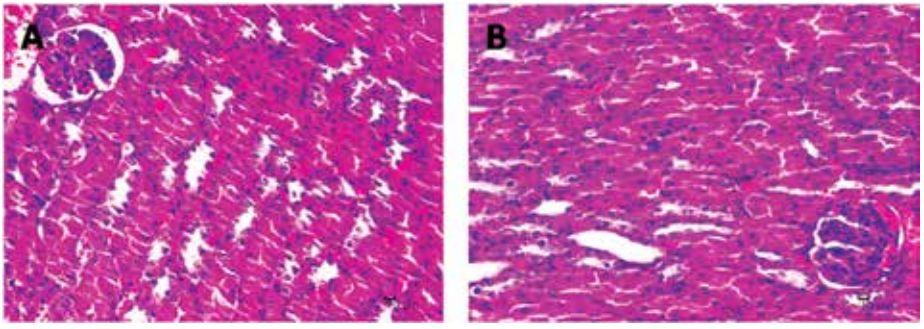

Fig 4-Pathological analysis of kidney in mice infected with MRSA.

Compared with blank group (A), the renal tissue lesions in model group (B) were not obvious. (400x)

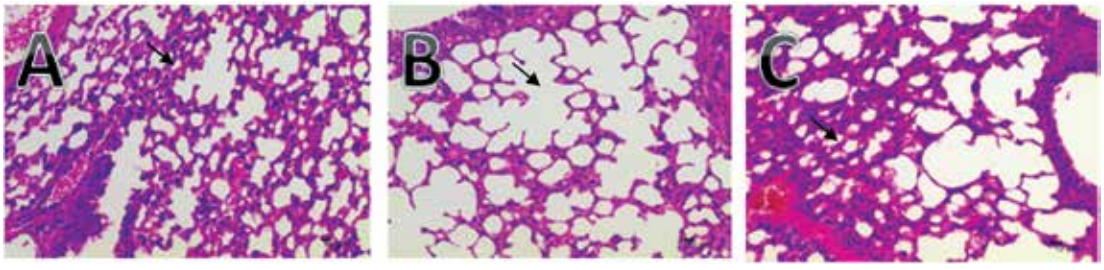

Fig 5-Histopathology of lung tissue from a methicillin-resistant *Staphylococcus aureus* (MRSA)-infected mouse treated with *Amomum tsao-ko* essential oil

The experimental protocol is shown in Fig 1 (post-treatment). Three mice infected with MRSA ($6.0\times10_{10}$ CFU/kg body weight) and treated with *A. tsao-ko* essential oil (0.93 g/kg body weight/day) were sacrificed and 4% formaldehyde-fixed 5 μm lung tissue sections were stained with hematoxylin and eosin and examined under a light microscope (400x magnification).

A: section from a mouse in blank control group; B: section from a MRSA-infected mouse; C: section from a mouse in preventive (post-treatment) group. Arrow indicates alveolus.

Although there are numerous studies on *in vitro* antibacterial activity of Chinese traditional medicine, fewer have reported *in vivo* efficacy (Harvey *et al*, 2015). In the present study, *A. tsao-ko* essential oil exhibited patent *in vivo* preventive and therapeutically efficacy against MRSA infection in a mouse model in a similar fashion to vancomycin through intragastric administration. Intramuscular injection of *A. tsao-ko* essential oil also exhibited satisfactory efficacy (unpublished).

In conclusion, the study shows *Amomum tsao-ko* essential oil demonstrated satisfactory *in vivo* preventive and therapeutic efficacies against methicillin-resistant *Staphylococcus aureus* infection in a mouse model. These findings suggest *A. tsao-ko* essential oil could be a potential candidate as a bactericidal against methicillin-resistant *S. aureus* in animals and possibly in humans.

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