Anti-microbial Effects In Vitro and In Vivo of *Alstonia scholaris*

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Abstract
*Alstonia scholaris* could be used as a traditional medicinal plant in China for the treatment of acute respiratory, which might be caused by respiratory tract infections. The investigation tested the anti-infective effects of total alkaloids extract (TA) from leaves of *A. scholaris*, and as a result, TA inhibited herpes simplex virus type 1 (HSV-1), respiratory syncytial virus (RSV) and influenza A virus (H1N1) in vitro respectively. In addition, the survival days of mice were prolonged, and the lung weights and mortality of mice were decreased significantly, after oral administrated TA in H1N1 and beta-hemolytic *streptococcus* infectious models in vivo respectively. The finding supported partly the traditional usage of *A. scholaris* in the treatment of respiratory infections.

Graphic Abstract

Keywords *Alstonia scholaris* · Total alkaloids · Acute respiratory infections · Anti-virus · Anti-bacteria

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We dedicate this paper to Prof. Zhou Jun in commemoration of his life-time contribution to researches in plant resource and phytochemistry.

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Abbreviations
TA Total alkaloids extract
ARIs Acute respiratory infections
H1N1 Influenza strains A/FM1/1/47
RSV Respiratory syncytial virus
IC$_{50}$ Half inhibitory concentration
TI Therapeutic index
CPE Cytopathogenic effect
TCID$_{50}$ Half of tissue culture infective dose
TC$_{50}$ Half toxicity
TC$_{0}$ Non-cytotoxic concentration
DMSO Dimethyl sulfoxide
MLD The minimum lethal dose
ED$_{50}$ Half of the effective dose
LD$_{50}$ Half of the lethal dose
RPMI Roswell Park Memorial Institute
FBS Fetal bovine serum
MLD The minimum lethal dose
MED The minimum effective dose

1 Introduction

Acute respiratory infections (ARIs) are the most common infections in humans, ranging from common cold through to very severe acute respiratory syndrome. ARIs are caused by over 200 viruses or bacteria [1]. Respiratory viruses seem to cause or serve as a co-pathogen in most cases of ARIs in epidemiologic studies. The specific viruses most frequently associated with acute bronchitis, in order of frequency of occurrence, are influenza, parainfluenza, respiratory syncytial virus (RSV), coronavirus, herpes simplex virus (HSV), adenovirus, and rhinoviruses [2]. The upper airway is a major ecological reservoir of bacterial species, bacterial infection ARIs approximately accounts for 15% of all patients, most of which are hemolytic streptococcus, followed by haemophilus influenzae, pneumococcus and staphylococcus, and occasionally gram-negative bacill [3].

Antiviral drugs and antibiotics are commonly used to treat ARIs in clinic, however, increasing drug resistance, toxicity, side effects, are formidable problems [4]. Therefore, alternative effective modalities for the treatment of respiratory infections are needed. And there is a growing recognition of the herbal medicine as its effectiveness has been investigated scientifically and a long history of native medicine [5].

Alstonia scholaris (L.) R. Br., an evergreen tree of Apocynaceae, is widely distributed in southwestern China, India, Thailand, Malaysia, Philippines, Africa, Australia, etc. [6]. In traditional medicine, the leaf of A. scholaris has been also used for treatment of many acute and chronic respiratory disorders for centuries [7–10]. The total alkaloids extract (TA) was obtained from the leaves of A. scholaris by extraction with ethyl acetate, and indole alkaloid content was more than 50%. Various laboratory-identified biological activities have been reported by our research group, including antitussive, anti-asthmatic, expectorant [11], analgesic, anti-inflammatory [12], anti-airway inflammation [13], anti-allergic asthma [14], anti-postinfectious cough [15], alleviating emphysema [16] and pulmonary fibrosis [17]. The anti-inflammatory effects were achieved by triggering the β$_2$ adrenergic receptor [18] and inhibiting the nuclear factor-κB expression [19]. In addition, preclinical safety evaluation results indicated that TA had a wide safety range in rats [20] or dogs [21] and no genotoxic effects [22]. Notably, the human pharmacokinetic results [23] were consistent with those of preclinical studies [14, 24], and the four principal alkaloids (picrinine, scholaricine, vallesamine, and 19-epischolaricine, Fig. 1) were all distributed in blood, thus ensuring credibility and validity for further study.

In addition, the chemical components of different parts of the herb were intensively investigated by our group [25–45]. In the leaves of this plant, picrinine, scholaricine, vallesamine, and 19-epischolaricine were proved to be the major bioactive alkaloids. TA extracted from A. scholaris leaves was approved as a new botanical drug (No. 2011L01436) by the China Food and Drug Administration, due to its strong pharmacological activity, low toxicity, and stable extraction process. Herein, we described antiviral (RSV, HSV-1, H1N1) and anti-bacterial (beta-hemolytic streptococcus) property in vitro and in vivo of TA, which might cause acute respiratory infections clinically.

Fig. 1 Four major alkaloids from A. scholaris
2 Results

2.1 Virus Infectivity

As shown in Table S1, the CPE of RSV and HSV-1 on Hep 2 and Vero cells at $10^{-13}$, $10^{-12}$, $10^{-11}$ and $10^{-10}$ groups were 25–70% of normal group, suggesting the RSV and HSV-1 preparations met the virulence criteria for a successful model of HSV-1 infection. The TCID$\text{}_{50}$ of RSV and HSV-1 were $10^{-12}$ and $10^{-11}$, respectively. In subsequent experiments, 100 folds TCID$\text{}_{50}$/mL of RSV and 10 folds TCID$\text{}_{50}$/mL of HSV-1 solution were selected.

2.2 The Effect of TA on Cell Viability

As shown in Table S2, the cell viability of TA on Hep 2 and Vero cells was pretty much the same, the TC$\text{}_{50}$ value and TC$\text{}_{0}$ was 18.75 and 6.25 mg/mL, respectively. They were all 1.25 (TC$\text{}_{50}$) and 0.313 (TC$\text{}_{0}$) mg/mL for ribavirinc and acyclovird.

2.3 TA Inhibited the RSV and HSV-1 Virus Replication In Vitro

The antiviral activity of TA extracted from A. scholaris was evaluated against RSV and HSV-1 by CPE inhibition assay (Table 1). Compared with control group, nearly all cells in RSV and HSV-1 alone group were dead (data not shown). Compared with RSV and HSV-1 alone group, TA exhibited satisfied anti-RSV and anti-HSV-1 activity, the un-infected cells in TA groups was significantly increased in a dose-dependent manner, and the IC$\text{}_{50}$ value was 3.13 and 1.56 mg/mL, the TI was 6 and 12, respectively. Positive control, ribavirinc and acyclovird also exhibited strong antiviral activity, with IC$\text{}_{50}$ values ranging from 0.02 (ribavirinc) to 0.01 mg/mL (acyclovid), and the TI value was 62.5 and 125, respectively.

2.4 TA Inhibited H1N1 Replication In Vitro

The chicken embryos were treated with TA in a dose-dependent manner to determine HI. As shown in Table 2, strong anti-H1N1 activity of TA was observed, with HI values (50 mg/mL: 1/160; 25 mg/mL: 1/320; 12.5 mg/mL: 1/480, respectively) comparable to that of control (Kugan granule, 1/160).

### Table 1: The inhibitory effect of TA against RSV and HSV-1 viruses in vitro

| Conc$^d$ | RSV | HSV-1 |
|---------|-----|-------|
| TA$^b$  | Conc$^c$ | Ribavirinc$^d$ | TA$^e$ | Conc$^f$ | Acyclovird$^g$ |
| 6.25    | 75  | 0.625 ND | 100  | 0.625 ND |
| 3.13    | 50  | 0.313 100 | 75   | 0.313 100 |
| 1.56    | 25  | 0.156 100 | 50   | 0.156 100 |
| 0.78    | 0   | 0.078 100 | 25   | 0.078 100 |
| 0.39    | 0   | 0.039 75  | 0    | 0.039 100 |
| 0.2     | 0   | 0.02  50  | 0    | 0.02  75  |
| 0.1     | 0   | 0.01  25  | 0    | 0.01  50  |
| 0.05    | ND  | 0.005 ND | 0    | 0.005 25  |

Ribavirinc and Acyclovird were the positive control

$^a$The concentration of TA (mg/mL)

$^b$The percentage of Hep 2 cells not infected by RSV viruses of TA (%)

$^c$The concentration of ribavirinc (mg/mL)

$^d$The percentage of Hep 2 cells not infected by RSV viruses of ribavirinc (%)

$^e$The percentage of Vero cells not infected by HSV-1 viruses of TA (%)

$^f$The concentration of acyclovird (mg/mL)

$^g$The percentage of Vero cells not infected by HSV-1 viruses of acyclovird (%)
2.5 TA Blocked H1N1-Infection in Mice

To further study the ability of TA to block H1N1 infectivity in vivo, we used a well-known mouse model of nasal H1N1 infection. Table S3 showed the LD50 of H1N1 was 1/200. Consequently, for the following experiments, mice were intranasally infected with 10 times LD50 of the virus. As shown in Table 3, infection with 10 times LD50 of H1N1 led to the death of all mice of model group within 14 days. Mice showed signs of piloerection, lethargy, weight loss and reduced food intake 3 days after H1N1 inoculation, and some of them died within 7–10 days of infection. Table 3 showed that the survival rate was 0% in the virus model group, but increased to 73.3%, 76.7% and 66.7% on 14 d in 50, 25 and 12.5 mg/kg of TA groups, respectively.

Meantime, the mean survival time of model group was 7.0 ± 2.3 days only, whereas, it was significantly prolonged to 12.27 ± 3.02, 12.37 ± 3.17 and 11.63 ± 3.61 days (all p < 0.01), the elongation ratio increased by 75.3%, 76.7 and 66.1% after the oral intervention of TA (50, 25 and 12.5 mg/kg).

In addition, weight of lung tissues was determined to verify whether TA could protect against H1N1 induced pulmonary injury. The lung weight in the model group (0.30 ± 0.035 g) was increased compared to the normal group (0.17 ± 0.016 g), indicating that H1N1 infection could cause swelling of lung tissues. Expectedly, it was showed a significant decrease of lung tissue weight in three doses of TA, and the average weights of lungs were 0.21 ± 0.015 g, 0.22 ± 0.015 g, and 0.23 ± 0.015 g compared with the virus-infected model group (0.30 ± 0.035 g, p < 0.01). Notably, the inhibitory effect of TA was comparable to control. Therefore, the minimum effective dose (MED) of TA against influenza A virus in Balb/C mice was 12.5 mg/kg and the TI was 64.

2.6 Protective Effect of TA Against Mice Infected with Beta-Hemolytic Streptococcus

As shown in Table S4, the MLD of beta-hemolytic streptococcus was determine to be 1 × 10^9 CFUs on female KM mice. Thus, for the follow-up experiments, mice were intraperitoneally infected with MLD of the pathogen.

Survivors of mice were recorded daily for 7 days post-challenge and shown as percentage of animal’s survival (Table 4). The mortality of model group was 100%, while treatment with TA increased the survival from 0 to 40% at all does (400, 200, 100, 50, 25, 12.5 mg/kg), in a non-dose dependent manner, and the ED50 value was 83.291 mg/kg. Hence, the TI value was calculated as 65.793 by the formula. Amoxicillin, the standard drug used in this experiment, reduced mice mortality by 0%.

3 Discussion

TA, an alkaloids extract from leaves of A. scholaris, commonly was used for treating the acute upper respiratory infection in traditional Chinese medicine. It was shown to reduce the numbers of neutrophil and leukocytes, induced by lipopolysaccharide infection and significantly attenuate histopathological changes and excessive secretion of inflammatory cytokines in an airway inflammation rat model.

In present paper, we reported the anti-infective activities of TA that may provide effective therapeutic approaches to the treatment of diseases such as infections of herpes simplex virus (HSV), respiratory syncytial virus (RSV) and influenza A/PR/8/34 (H1N1) virus. HSV, a double stranded DNA virus belonging to the herpesviridae family, involves two human antigen types, HSV-1 and HSV-2 [46]. Clinical manifestations of this viral infection range from benign to severe and life-threatening syndromes in immunocompromised patients. In present study, the inhibitory effects of TA against HSV-1 were manifested as a marked reduction in cytopathic

### Table 3 The inhibitory effect of TA against H1N1 virus in vivo

| Group   | N  | Survivors | Survival rate (%) | Mean survival time | Elongation ratio (%) | Lung weights (g) | Inhibition ratio (%) |
|---------|----|-----------|-------------------|-------------------|----------------------|----------------|---------------------|
| Normal  | 30 | 30        | –                 | 14 ± 0.0          | –                    | 0.17 ± 0.016   | –                   |
| Model   | 30 | 0         | –                 | 7.00 ± 2.30       | –                    | 0.30 ± 0.035   | –                   |
| Control | 30 | 20        | 66.7*             | 12.03 ± 2.94**    | 71.8                 | 0.21 ± 0.017*  | 69.2                |
| TA-50   | 30 | 22        | 73.3*             | 12.27 ± 3.02**    | 75.3                 | 0.21 ± 0.015*  | 69.2                |
| TA-25   | 30 | 23        | 76.7*             | 12.37 ± 3.17**    | 76.7                 | 0.22 ± 0.015*  | 61.5                |
| TA-12.5 | 30 | 20        | 66.7*             | 11.63 ± 3.61**    | 66.1                 | 0.23 ± 0.015*  | 53.9                |

Values represent the mean ± standard deviations (SD) of three independent experiments for anti-H1N1 assay. TA was dosed from 12.5 mg/kg to 50 mg/kg. ▲▲p < 0.01 vs. the normal group; *p < 0.05 or **p < 0.01 vs. the model group.
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...effect of Vero, the IC50 and TI value were 1.56 mg/mL and 12.

Human respiratory syncytial virus (RSV), an Orthopneumovirus belonging to the Pneumoviridae family, is one of the most important pathogens causing severe acute lower respiratory infections [47]. The clinical manifestations after RSV infection range from a mild upper respiratory tract infection to severe life-threatening lower respiratory tract involvement such as bronchiolitis, pneumonia, and croup, together with some common symptoms including fever, rhinorrhea, cough, and wheezing [48], which are not readily distinguished from those of other common respiratory virus infections. As expected, the replication of RSV was inhibited by the intervention of TA in vitro, the IC50 and TI against RSV were 3.13 mg/mL and 6, respectively.

Influenza A virus, one of the most infectious influenza viruses, contains an eight-segmented negative sensed RNA that codes a total of 14 different proteins [49]. These viral proteins facilitate the virus replication inside the target cell, exploit the host immune system and intracellular pathway store plicate and prompt the releasing of newly formed viral particles from the infected cell [50]. It has been reported that the fatal consequence of the influenza is eminently associated with acute pneumonia which could destabilize the atherosclerotic plaque in the arteries, increasing the risk of heart attack and stroke [51]. The H1N1 virus replication was greatly suppressed in the presence of TA, and the hemagglutination titer was significantly reduced from 1/1280 to 1/160.

In vivo testing without doubt is one of the recognized, if not the most important, essential links between in vitro sensitivity testing and clinical studies. In fact, several guide lines for the clinical evaluation of efficacy and toxicity of anti-infective drugs explicitly require experimen- tational evaluation of new compounds (or novel combinations or therapeutic modalities) in animals as prerequisites for clinical trials [52, 53]. Following, we investigated the protective effect of TA against the H1N1 virus in a mice model by intranasal inoculation virus suspension. Importantly, oral administration of 50, 25, 12.5 mg/kg TA possessed effective antiviral activity in infected-mice, conferring 73.3%, 76.7% and 66.7% protection from death against H1N1, which was superior to those of Kugan granule (66.7%). TA also prolonged survival time and decreased lung weights in virus-infected mice. Taken together, the antiviral effects of TA against influenza virus were demonstrated in this study, which agrees with findings of previous studies [54].

Moreover, upper respiratory infection caused by virus can easily lead to secondary infections caused by bacteria. Beta-hemolytic streptococcus is one of the major human pathogens that cause both community- and hospital-acquired infections [55]. The infections caused by it ranged from the relatively mild superficial skin purulent inflammation to the more serious conditions as respiratory tract infection, epidemic pharyngitis, neonatal sepsis, bacterial endocarditis, scarlet fever, rheumatic fever and glomerulonephritis [56]. First of all, the upper respiratory tract mucous membrane was damaged by beta-hemolytic streptococcus and its discharge toxins, with the symptoms of pharyngeal hyperemia, edema. Furtherly, local inflammatory cell infiltration and seepage resulted in redness and swelling of the pharynx, tonsil, soft palate and even uvula. Eventually, tonsil and soft palate mucosa often undergo necrosis and develop into purulent pharyngeal buccal inflammation and tonsils.

Dose bacteria pathogenicity experiments were conducted to determine the MLD of the pathogen beta-hemolytic streptococcus infection in mice. The MLD50 was 1 × 109 CFUs in female mice. Therefore, for subsequent experiments, mice were infected with MLD of the pathogen by intraperitoneal injection. In particular, TA, at all the doses tested, effectively protected the mice from infected by beta-hemolytic streptococcus and increased animal survival rate, thus establishing

Table 4 Protective effect of TA against mice infected with beta-hemolytic streptococcus

| Group        | N  | Day 4 | Day 5 | Day 6 | Day 7 | Survival rate (%) |
|--------------|----|-------|-------|-------|-------|-------------------|
| Normal       | 20 | 0     | 0     | 0     | 0     | 100              |
| Model        | 20 | 11    | 8     | 1     | 0     | 0                |
| Amoxicillin -300 | 20 | 0    | 0     | 0     | 0     | 100              |
| TA-400       | 20 | 8     | 3     | 1     | 0     | 40               |
| TA-200       | 20 | 11    | 2     | 0     | 0     | 35               |
| TA-100       | 20 | 12    | 3     | 0     | 0     | 25               |
| TA-50        | 20 | 16    | 2     | 0     | 0     | 10               |
| TA-25        | 20 | 14    | 3     | 0     | 0     | 15               |
| TA-12.5      | 20 | 17    | 1     | 0     | 0     | 10               |

N: Total number of animals in three experiments
Amoxicillin as the positive control. The dose of amoxicillin was 300 mg/kg, and TA was dosed from 12.5 mg/kg to 400 mg/kg
the potential in vivo antibacterial activity of TA and further supporting its traditional use in the treatments of ARIs.

4 Materials and Methods

4.1 Plant Materials

Leaves of *A. scholaris* were purchased from Datang–Hanfang Medicine Co., Ltd. (Pu’er, China). The plants were collected in 2006 in Pu’er City, Yunnan Province, People’s Republic of China. Dr. Xiao–Dong Luo of the Kunming Institute of Botany, Chinese Academy of Sciences, identified the plants, and the plant name was checked against [http://www.theplantlist.org](http://www.theplantlist.org). A voucher specimen (no. Luo20060407) was deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Chinese Academy of Sciences.

4.2 Preparation of Alkaloids

Dried and powdered leaves of *A. scholaris* were extracted with 90% ethyl alcohol under reflux conditions (3 h × 4 times) at room temperature, and the solvent was evaporated *in vacuo* to obtain the ethanolic extract. The ethanolic extract was dissolved in 0.3% aqueous HCl solution and filtered; the residue was the nonalkaloid fraction. Then, the acidic solution, adjusted to pH 9–10 with 10% aqueous ammonia, was extracted with ethyl acetate to obtain the total alkaloids (TA) fraction (batch no. 20070512, 20111101).

4.3 Chemicals, Viruses, Bacteria and Cells

Acyclovir and ribavirin were selected to be antiviral control against HSV-1 and RSV and provided by Tianjin Pharmaceutical Jiaozuo Co, Ltd (Tianjin, China). Kugan granule, a launched traditional Chinese medicine formula, was used to be antiviral control against H1N1, and provided by Qingdao Guofeng Pharmaceutical Co. Ltd (Qingdao, Shandong). Amoxicillin was chosen as an antibacterial control and produced by Zhuhai Federal Pharmaceutical Co. Ltd (Hongkong, China).

The influenza strains A/FM1/1/47 (H1N1) and herpes simplex virus type 1 strain sm44 (HSV-1) were obtained from National Institute for Viral Disease Control and Prevention, China Center for Disease Control and Prevention. Respiratory Syncytial Virus (RSV) was provided by West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University. Hep2 and Vero cells were purchased from Conservation Genetics CAS Shanghai Cell Bank. Specific-pathogen-free embryonated eggs were denoted by West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University. Standard strain beta-hemolytic *streptococcus* was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA).

4.4 Animals

Specific-pathogen-free, female, Balb/C and KM mice (weight: approximately 20 g) were purchased from West China Medical Laboratory Animal Center, Sichuan University (license number SCXK [Chuan] 3-042-2010). Our experiments were approved by the Institutional Animal Care and Use Committee of the Sichuan University and performed according to the international rules concerning animal experiments and the internationally accepted ethical principles for the use and care of laboratory animals.

4.5 Virus Virulence and Cytotoxicity Assay

The anti-RSV and anti-HSV-1 activities of TA were determined by the titer reduction assay as previously described [57]. Briefly, Hep2 (to RSV) and Vero cell monolayers (to HSV-1) were co-incubated with a series of virus concentrations from $10^{-3}$ to $10^{-13}$. The cytopathogenic effect (CPE) was observed and half of tissue culture infective dose (TCID$_{50}$) was calculated according to Reed & Muench formula [58].

The half toxicity (TC$_{50}$) and non-cytotoxic concentration (TC$_{0}$) of TA against Hep2 and Vero cells were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previous [59].

4.6 Anti-HSV-1 and—RSV Activity Assay In Vitro

Methods was referred to the previous with minor modifications [60], TA was suspended in 1% dimethylsulfoxide/sterile water for injection. Equivalent of 100 folds TCID$_{50}$/mL of RSV for Hep2 cells and 10 folds TCID$_{50}$/mL of HSV-1 for Vero cells were added to the cell cultures which were then incubated at 37 °C in a 5% CO2 atmosphere, and the supernatant was removed after incubation for 2 h. Cells were then treated with serial concentrations of TA diluted from TC$_{50}$ to 1/64 TC$_{50}$ (mg/mL) or with two-fold serial dilutions of ribavirin and acyclovir (from 1/2 TC$_{50}$ to 1/128 TC$_{50}$) used as the positive control. All wells were then observed under a light microscope to determine the virus-induced cytopathic effect (CPE) at day 5 after the treatment. The concentrations of the test articles that reduced CPE by 50% (IC$_{50}$) were determined. And the therapeutic index (TI) value was calculated as the ratio TC$_{50}$/IC$_{50}$ [61].

4.7 Effect of TA on H1N1-Infection In Vitro

Methods was referred to the previous with minor modifications [60], TA was suspended in 1% dimethylsulfoxide/sterile water for injection. Equivalent of 100 folds TCID$_{50}$/mL of RSV for Hep2 cells and 10 folds TCID$_{50}$/mL of HSV-1 for Vero cells were added to the cell cultures which were then incubated at 37 °C in a 5% CO2 atmosphere, and the supernatant was removed after incubation for 2 h. Cells were then treated with serial concentrations of TA diluted from TC$_{50}$ to 1/64 TC$_{50}$ (mg/mL) or with two-fold serial dilutions of ribavirin and acyclovir (from 1/2 TC$_{50}$ to 1/128 TC$_{50}$) used as the positive control. All wells were then observed under a light microscope to determine the virus-induced cytopathic effect (CPE) at day 5 after the treatment. The concentrations of the test articles that reduced CPE by 50% (IC$_{50}$) were determined. And the therapeutic index (TI) value was calculated as the ratio TC$_{50}$/IC$_{50}$ [61].
inhibitory activity of influenza virus H1N1 was determined by
chicken embryo method and the virus titers was 1/1280. All
embryonated eggs except the normal group were injected
with 0.1 mL H1N1 virus (60 U/mL) via allantoic cavity
inoculation before treatment. Then the eggs inoculated virus
were randomly divided into 5 groups. The embryonated eggs
in model group received 0.1 mL of 1% DMSO, the embryo-
nated eggs in the positive group received 0.1 mL of control;
group 3 to 5 were administrated different concentrations of
TA treatment, and finally, the normal group was also inocu-
lated with 0.1 mL of 1% DMSO. The concentration of TA
was non-toxic to chicken embryos confirmed by a prelimi-
nary experiment (data not shown). Allantoic fluid of chicken
embryo were collected from each group at 48 h post infec-
tion, and the hemagglutination titer (HI) was determined by
hemagglutination assay with 1% chicken red blood cell. The
virus titer of H1N1 declined by one dilution was considered
to be effective, and significant effect by two dilutions, when
compared the model group.

4.8 Effect of TA on H1N1-Infection in Mice

A preliminary examination was carried out before the formal
experiment began to determine the virulence of H1N1 virus
on mice. For infectious experiment, 60 eight weeks BALB/c
female mice were divided into six groups including nor-
mal (mice without viral infection); model (infected mice); control (Kugan granule, 960 mg/kg) and TA-treated groups
(50, 25, 12.5 mg/kg). The choice of the doses used in this
experiment was based on the pilot study [63] and the results
from acute oral toxicity study [20]. The treatment started
2 days before viral challenge. Accordingly, treatment of the
mice with TA or positive control started two days before
infectious. For the normal and model group, the mice were
given 1% DMSO instead. Subsequently, all mice except the
normal group were challenged with 10 times LD$_{50}$ of H1N1
by intranasal inoculation of 30 μL of virus suspension. An
equal volume of intranasal saline was substituted for the nor-
mal group. Following viral, the treatment was repeated daily
for 14 consecutive days and survival animals were recorded
for 14 days, and the lungs were then collected and weighted.

4.9 Anti-Beta-Hemolytic Streptococcus Assay

in Mice

The minimum lethal dose (MLD) was regarded as the
minimum amount of infectious bacteria that caused all
the infected animals to die within 1–2 days after the intra-
peritoneal injection of beta-hemolytic streptococcus sus-
pension [64]. Briefly, forty-five female KM mice were
randomly divided into nine groups (each group of five).
Then, forty animals in different groups were administrated
intraperitoneally with beta-hemolytic streptococcus suspen-
sion ($10^{10}$–$10^{3}$/mL) at a volume of 0.5 mL/mouse. Normal
animals were given the same volume of saline solution.

As for the protection against beta-hemolytic streptococcus
in vivo, the mice were divided into nine groups of ten
mice each: normal (mice without bacterial infection); model
(infected mice); amoxicillin-treated group (positive control,
300 mg/kg) and six TA-treated groups (400, 200, 100, 50,
25, 12.5 mg/kg). The oral treatment was administered two
days prior to infection. For normal and model groups, 1%
DMSO was administered by oral gavage. Subsequently, the
bacteria-infected groups except normal group were inocu-
lated intraperitoneally with MLD of 0.5 mL of beta-hemo-
lytic streptococcus suspension. For the normal group, the
mice were given the same volume saline water in place of
the bacterial suspension. Following bacterial challenge, the
treatment was repeated one time after infectious. The clinical
signs and surviving animals were recorded daily till 7 days
post-infection. Half of the effective dose (ED$_{50}$) was calcu-
lated using Bliss method, and then the therapeutic index (TI)
at each test was determined using the following formula:
TI = LD$_{50}$/ED$_{50}$ (LD$_{50}$: half of the lethal dose, 5.48 g/kg).

4.10 Statistical Analysis

Data from the experiment are expressed as the mean ± stand-
ard deviation of the mean. Two-tailed Mann–Whitney U
test was used for comparing between two groups, one-way
analysis of variance with least significant difference or
Kruskal–Wallis analysis with Dunn post-test for multiple
comparisons and chi-square test, in which nonparametric
analyses were appropriate. Analyses were performed using
the Prism software (GraphPad, San Diego, CA, USA). A
p-value < 0.05 denoted statistical significance in all analyses.

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Compliance with Ethical Standards

Conflict of interest There are no known conflicts of interest associated
with this publication.

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