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Pharmacodynamic study in multi-animal models on the efficacy and optimal dosage of antiviral oral liquid for children

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Abstract

Antiviral Oral Liquid (AOL) is an adult medicine in the Chinese Pharmacopoeia used to treat upper respiratory infections such as influenza. It has shown promising clinical efficacy in relieving flu-like symptoms such as fever, inflammation, and pharyngalgia both in adults and children. However, the instruction manual does not specify the exact usage and dosage of AOL for children. In this article, we set 6 dosage ranges: 0.2, 0.5, 0.7, 0.9, 1.1, 1.4 mL/kg/d, according to its dosage for adults and the conversion method between adult and children dosage. And six animal models were used to evaluate the effectiveness of AOL in different doses. The results indicated that AOL could reduce the lung index, virus load, and expression of proinflammatory cytokines in the lung. AOL could improve pathological changes and prolong the survival time of mice infected by the Influenza A virus (H1N1) A/PR/8/34 strains at 0.5–0.9 mL/kg/d concentrations in different degrees. The four dose groups of 0.7–1.4 mL/kg/d could significantly inhibit the ear shell swelling caused by xylene and reduce the rabbit body temperature induced by lipopolysaccharide ($P < 0.01$, $P < 0.05$). All the five dosage groups of 0.2–1.1 mL/kg/d could inhibit the increase of peritoneal capillary permeability induced by glacial acetic acid ($P < 0.01$). AOL at 0.7 and 0.9 mL/kg/d reduced the painful writhing times in young mice induced by glacial acetic ($P < 0.01$). These results indicated that the optimal dose of AOL in antiviral, antipyretic, anti-inflammatory, and analgesic effects is 0.7 mL/kg/d.

Keywords: antiviral oral liquid; clinical application in children; antiviral activity; anti-inflammatory activity; analgesic activity

Author contributions

Sun J, Wang L and Cui XL conceptualized this study. Sun J, Zhao RH, Bao L and Guo SS designed and performed the experiments. Cao JM, Zhang L, Wang L, Sun J and Dai Z analyzed the data. Cao JM, Xu YL, Cui XL, Wang L and Sun J prepared the draft and final version of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

AOL, antiviral oral liquid; ELISAs, enzyme-linked immunosorbent assays; TCM, traditional Chinese medicine; HPLC, high-performance liquid chromatography; ICR, Institute of Cancer Research; XFK, Xiaoer Feire Kechuan; LPS, lipopolysaccharide; ANOVA, analysis of variance.

Citation

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Highlights

Multi-animal models were performed to evaluate the effectiveness of antiviral oral liquid in different doses. Indicated that the optimal dose of antiviral oral liquid in antiviral, antipyretic, anti-inflammatory, and analgesic effects is 0.7 mL/kg/d.

Medical history of objective

Antiviral oral liquid originated from the "*Treatise on Cold Damage Diseases*" by Zhang Zhongjing and improved on the basis of the Chinese medicine prescriptions "White Tiger Decoction" and "Qingwen Baidu Decoction", which has more than 1,000 years of successful experience. It is a combination of classic prescriptions and modern medical science and technology. In current studies, modern pharmacological research has shown that antiviral oral liquid has effects. It is widely used in clinic to prevent and treat cold, influenza and other diseases caused by virus infection such as hand, foot and mouth disease, herpangina, conjunctivitis and so on.

Introduction

Respiratory viral infections such as the severe acute respiratory syndrome in 2003, H1N1 Influenza in 2009, and the coronavirus disease at the end of 2019 tend to result in grievous pandemics that seriously threaten human health. It caused significant economic losses and even influence global politics and history. Influenza is an acute contagious respiratory disease and is popular almost every year. There are three main types of influenza viruses capable of causing infection in humans. Influenza A virus H1N1 is the dominant type causing seasonal epidemics because of its high susceptibility to antigenic variation [1]. The symptoms of influenza can run from mild to severe; the hallmark of infection is abrupt onset of fever, headache, fatigue, cough, sore throat, and myalgias [2]. But in some hypoimmunity populations, especially in infants and children, influenza can be complicated by acute pneumonia, febrile seizures, dehydration, or encephalopathy, and lead to increased morbidity and mortality [3]. Although scientific advances have been made in understanding the aetiological attributes of the Influenza A virus and the characteristics of physiological structure and immune function in children, it is challenging to resolve the urgent problem of the shortage of antiviral medicines for children and the development of potential effective control measures [4–6].

Up to 2020, only a few drugs have been approved by the Food and Drug Administration for viral infectious respiratory diseases, mainly including broad-spectrum antiviral drugs (e.g., interferon), anti-RNA viral drugs (e.g., oseltamivir), and anti-DNA viral drugs (e.g., nucleoside analogs) [7]. However, children are not included in preclinical studies of most existing marketed drugs, and the studies are lacking in the safety, effectiveness, and optimal dosage for children. Children of different ages are different in terms of absorption, distribution, metabolization, and excretion. Therefore, the medical treatment of pediatric diseases is much more complicated than that of adults. The treatment should be determined following the characteristics and specific conditions of the child in different periods. Therefore, identifying the appropriate usage and dosage is of great significance for the clinical treatment of children's diseases.

Oseltamivir is the most common drug used to treat influenza in children. It is a neuraminidase inhibitor, which prevents the virions from releasing from the infected cells and inhibits the diffusion of the virus in the respiratory tract [8]. However, the mutations in the genetic characteristics of neuraminidase in Flu viruses can induce the resistance of oseltamivir. In addition, the therapeutic effect of oseltamivir is related to the time of taking the drug, which is generally given within 48 hours of illness onset. Besides, oseltamivir has been associated with the development of gastrointestinal adverse events (i.e., vomiting, nausea and diarrhea) and transient neuropsychiatric events (i.e., self-injury or delirium) in children and adolescents [9, 10]. Therefore, the clinical application of oseltamivir is severely limited and its safety risks are increased.

In the process of fighting the epidemic, traditional Chinese medicine (TCM) attracted sufficient attention of many medical experts in the world. TCM has been used for thousands of years in the prevention and treatment of infectious diseases and accumulated a great deal of experience and research results in the prevention and treatment of influenza. Considering the natural non-toxicity, immune regulation, and multi-target characteristics, the use of TCM in the treatment of children's respiratory viral infectious diseases has broad prospects. Antiviral oral liquid (AOL) is included in the Chinese Pharmacopoeia, and the prescription is developed based on the "*Treatise on Febrile Disease*" by the famous traditional Chinese medicine doctor Zhang Zhongjing. The prescription contains nine traditional Chinese herbs, including *Radix isatidis*, Gypsum, *Reed rhizome*, *Rehmannia glutinosa* (Gaetn.) Libosch. ex Fisch. et Mey., *Curcuma longa* L., *Anemarrhena asphodeloides*, *Acorus calamus* L., *Pogostemon cablin* (Blanco) Benth., and *Forsythia suspense*. AOL is widely used in clinics to prevent and treat colds, influenza and other diseases caused by virus infections such as hand, foot and mouth disease, herpangina, conjunctivitis and so on.

AOL obtained the new drug certificate in 1989 and showed definite clinical efficacy in both adults and children in more than 30 years of clinical application. It could relieve cold symptoms such as fever, headache, sore throat, runny nose, etc. The dosage of AOL for adults was 10 mL/time, 2–3 times/day [11]. However, the dosage of AOL for children is unclear. This study aims to explore the effectiveness and optimal dosage of AOL for children and provide a basis for clinical application. Based on four conversion methods (age, body weight, body surface area and ratio), the possible medication range for children aged 1–12 years was set according to the adult dosage [12, 13]. The medication range for children was further converted into the dosage for young mice and rabbits [14]. In this study, the antiviral, anti-inflammatory, antalgic, and antipyretic effects of AOL at different dosages were determined in muti-animal models to evaluate the efficacy and optimal dosage of AOL for children.

Materials and methods

Medication

AOL (Lot. 202005109) was manufactured by Hangzhou China Resources Laotongjun Pharmaceutical Co., Ltd. (Hangzhou, China). The drugs used and their manufacturers are as follows: Oseltamivir phosphate capsules (Lot. 0221901008, Yichang Dongyangguang Pharmaceutical Co., Ltd., Yichang, China), Xiaoer Feire Kechuan (XFK) oral liquid (Lot. 201909007, Heilongjiang Sunflower Pharmaceutical Co., Ltd., Harbin, China), Ibuprofen suspension (Lot. 191218223, Shanghai Johnson Pharmaceutical Co., Ltd., Shanghai, China).

Dose conversion

The dosage range for children was converted according to age, weight, ratio, or body surface area based on the adult dosage [12, 13]. Then, the dosage for children was further converted into those for young mice and rabbits according to the classic animal dose conversion formula [14]. The dosage range for children aged 1–12 years that was converted in accordance with four conversion methods was 0.4–0.8 mL/kg/d. In animal model experiments, a total of 6 dosage ranges: 0.2, 0.5, 0.7, 0.9, 1.1, 1.4 mL/kg/d were set.

Quality control of AOL

Preparation of the sample. AOL preparation: The preparation method refers to the Pharmacopoeia of the People's Republic of China [15]. In brief, 25 mL of AOL was partitioned six times with ethyl acetate (6 × 25 mL). Then, the ethyl acetate fraction was combined and concentrated under vacuum to yield residue. The residue was dissolved in 70% methanol and then accurately diluted to 10 mL with

a 70% methanol aqueous solution.

Reference solution preparation: A standard reference solution containing the two components (0.02 mg/mL (R, S)-goitrin, 0.06 mg/mL forsythin) was prepared in a 70% methanol aqueous solution.

Forsythin solution preparation: An appropriate amount of forsythin reference substance was measured, accurately weighed, and added with 70% methanol to make a solution containing 75 μg/mL forsythin.

Characteristic map determination of AOL. The method was following the Pharmacopoeia of People's Republic of China [13]. The high-performance liquid chromatography (HPLC) conditions were as follows: column, YMC Hydrosphere C18, 4.6 × 250 mm, 5 μm; flow rate, 1 mL/min; UV spectra wavelength, 236 nm; mobile phase, acetonitrile (A), 0.01% phosphoric acid water (B); gradient elution program, started from 7% A altered to 18% A, 0–22 min; 18% A, 22–29 min; started from 18% A altered to 23% A, 29–31 min; 23% A, 31–40 min; started from 23% A altered to 40% A, 40–53 min; 40% A, 53–60 min; and started from 40% A altered to 7% A, 60–65 min. The temperature of the column oven was 30 ˚C, and 10 μL sample (AOL preparation and reference solution) was injected into the system.

Determination of forsythin content in AOL. The method was also in accordance with the Pharmacopoeia of People's Republic of China [15]. The HPLC conditions were as follows: flow rate, 1 mL/min; UV spectra wavelength, 277 nm; and mobile phase, acetonitrile (A): water (B) = 23:77.

Virus

The influenza A virus (H1N1) A/PR/8/34 strain was obtained from the American Type Culture Collection (Manassas, VA, USA).

Animals

Institute of Cancer Research (ICR) mice (3 weeks, 14 ± 1 g, half male and half female, specific pathogen free) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. (Beijing, China). The mouse experiments were performed in the ABSL-2 biosafety laboratory of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences (Beijing, China). Five-week-old rabbits were purchased from Beijing Jinmuyang Experimental Animal Breeding Co., Ltd. (Beijing, China). The rabbit experiments were performed in Beijing Jinmuyang Experimental Animal Breeding Co., Ltd. (Beijing, China). All animals were maintained in a standard laboratory conditioned at a temperature of 25 ± 2 ˚C with 50–55% relative humidity and a 12-hour light/dark cycle. The experimental protocol in this study was ethically reviewed and approved by the Animal Welfare Ethics Committee of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, and the record number is 2020D011. All animal experimentation protocols were conducted in strict accordance with the Guidelines for the Care of Laboratory Animals (Ninth Edition).

Pneumonia induced by influenza virus infection in mice

One hundred mice were randomly divided into ten groups including: normal group, model group, positive groups of oseltamivir, positive groups of XFK oral liquid, and 6 AOL groups at the dosages of 0.2, 0.5, 0.7, 0.9, 1.1 and 1.4 mL/kg/d, with ten mice in each group. Except for the normal group, the mice were lightly anesthetized and infected with 35 μL PR8 influenza virus intranasally. Mice in the normal group were intranasally lightly anesthetized and administered saline via nasal drip. The mice were given an intragastric administration once a day for four consecutive days. When taking samples, Mice were fed normal mouse chow and water ad libitum and were maintained under standard conditions with air filtration. Quick and humane sacrifice of a mouse by cervical dislocation.

Body weight and lung weight were recorded and the lung index and lung index inhibition rates were calculated as follows: Lung index = Wet lung weight (g)/Body weight (g) × 100%; Lung index inhibition rate = (Model group lung index − Drug administration group lung index)/(Model group lung index − Normal group lung index) × 100%.

Histopathological examination of lung tissues

Four days post-infection, lung tissues that were fixed in 4% paraformaldehyde fixing solution were dehydrated, embedded in paraffin and sliced into 5 μm sections. The sections were stained with hematoxylin and eosin reagents.

Enzyme-linked immunosorbent assays (ELISAs)

Four days post-infection, lung samples were homogenized, and levels of IL-6 and TNF-(Cloud-clone crop, China) were determined by ELISA as per the manufacturer's instructions.

RNA isolation and RT-PCR

The lung tissues were stored at −80 ˚C. Lung tissue RNA extracted by TRIzol Reagent according to the manufacturer's instructions (Invitrogen). Quantification of viral load was performed according to the manufacturer's instructions (Liferiver, China). Standard curves were generated using the positive controls in the kit. PCR products were analyzed using QuantStudioTM Design & Analysis software v1.5.1 [16].

Death induced by influenza virus infection in mice

We grouped 180 ICR mice as described above with 20 mice in each group but without a normal control group. The details of the virus infection and administration are the same as described above. We observed the death of the mice within 2 weeks after infection, then calculated the mortality, average survival days, and life extension rate as follows: Mortality = (Number of deaths/Total mice) × 100%; Life extension rate = (Survival time in days of drug administration group-Survival time in days of the model control group)/(Survival time in days of the model control group) × 100%

Anti-inflammatory experiment

The anti-inflammatory activity of AOL was determined in xylene-induced ear edema mice [14]. Briefly, 100 ICR mice were divided into ten groups consisting of 10 mice. Ibuprofen and XFK oral liquid were used as positive drugs. Each mouse was given an intragastric administration once a day for 3 consecutive days. 1 h after the last administration, 30 μL xylene was evenly smeared on the front and back of the right auricle of the mice. Then the weight difference between the two ears was recorded as the degree of edema, which is an indicator of inflammation. Edema inhibition rate (%) = (Degree of edema in the model group-Degree of edema in drug administration group)/(Degree of edema in the model group) × 100%.

The anti-inflammatory activity of AOL was further verified in mice model of the increased permeability of abdominal capillaries induced by acetic acid [17]. Briefly, 100 ICR mice were grouped as described above. Each group was given an intragastric administration once a day for three consecutive days. 1 h after the last administration, each mouse was intraperitoneally injected with 0.15 mL of 1% acetic acid solution, and the tail vein was injected with 0.2 mL of 0.5% Evans blue solution. Then, the abdominal cavity fluid was collected 20 minutes later and centrifuged at 1000 r/min for 5 min. The supernatant was extracted, and the absorbance was measured at 590 nm with a microplate reader.

Analgesic test

The analgesic effect of AOL was detected in young mice writhing test induced by acetic acid [18]. Briefly, 90 ICR mice were grouped as described above but without a normal control group, and each group consisted of 10 mice. Each drug administration group was given an intragastric administration once a day for 3 consecutive days, and the model group was given distilled water under the same conditions. Then 1% acetic acid was injected directly into the abdomen to stimulate the peritoneum and cause pain, resulting in a writhing reaction in mice, which was manifested as abdominal concave, trunk and hind limb stretching, hip lifting, etc. The number of writhing times of the mice in the following 15 min was recorded.

LPS-induced fever experiment

The antipyretic effect of AOL was investigated in the fever rabbits

induced by lipopolysaccharide (LPS) [17]. Briefly, the body temperature of rabbits was monitored 2 days before and on the day of the experiment. We selected rabbits whose body temperatures were at 39.4–40.0 ˚C and fluctuated within 0.5 ˚C. Then, 60 rabbits with qualified body temperatures were selected and divided into normal control group, model group, ibuprofen group, XFK oral liquid group, and AOL dosage groups of 0.2, 0.5, 0.7, 0.9, 1.1 and 1.4 mL/kg/d according to the basal body temperature. Each group was composed of 6 rabbits. Except for the control group, all the groups received an auricular vein injection of 250 ng/kg LPS. Then drugs were administrated after 1 h at a dose of 5 mL/kg. The body temperatures at 1, 2, 3, and 4 h after administration were measured and the body temperature difference was calculated.

Pharmacokinetic study

C57BL/6J mouse was put into a metabolic cage and fasted for 12 h, but free to water before dosing. Mice were given AOL by intragastric administration at a dose of 1.4 mL/kg/d. Blood was taken from the tail vein post administration for 1, 12, and 24 h. Plasma was centrifugated at 3000 rpm for 10 min. The urine and feces samples were collected for 12 h before and 24 h after drug administration, respectively. All the samples are analyzed by HPLC.

Statistical analyses

The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or Kruskal-Wallis test with SPSS. One-way ANOVA was used for comparisons between groups, and the least significant difference method was used for multiple comparisons when variances were equal. The homogeneity of variance test was used for unequal variances. Two-way repeated measures ANOVA was used for mean differences in body temperature between groups between time points. (SPSS 17.0, IBM Corporation, Armonk, NY, USA). *P* values < 0.05 were considered to be statistically significant. All data are expressed as the mean ± SD ($\bar{x}$ ± s).

Results

Quality control of AOL

Under the requirements of the Pharmacopoeia of People's Republic of China, the characteristic map of AOL was determined, and the results are shown in Figure 1 [13]. Seven characteristic peaks were observed in the characteristic map of AOL. Peak 2 is (*R*, *S*)-goitrin, and peak 6 is forsythin. In addition, we calculated the relative retention time of the 6 characteristic peaks, and they all met the requirements of the Pharmacopoeia of the People's Republic of China [13]; that is, the relative retention time of peak 1 was within ± 5% of the specified value (0.58), and the relative retention of the remaining characteristic peaks was within ± 8% of the specified value (1.00, 2.38, 2.61, 2.65, and 4.94).

To further test the quality of AOL, we took three batches of AOL samples and compared them with the standard products to determine the content of forsythin in AOL. In accordance with the requirements of the Pharmacopoeia of People's Republic of China, the content of forsythin (the retention time was 47.862 min) in AOL should not be < 25 μg/mL [13]. The content of forsythin was determined to be 137.5 ± 5.89 μg/mL in three batches of AOL samples (Figure 2). The experimental results indicate that AOL is qualified and suitable for further research.

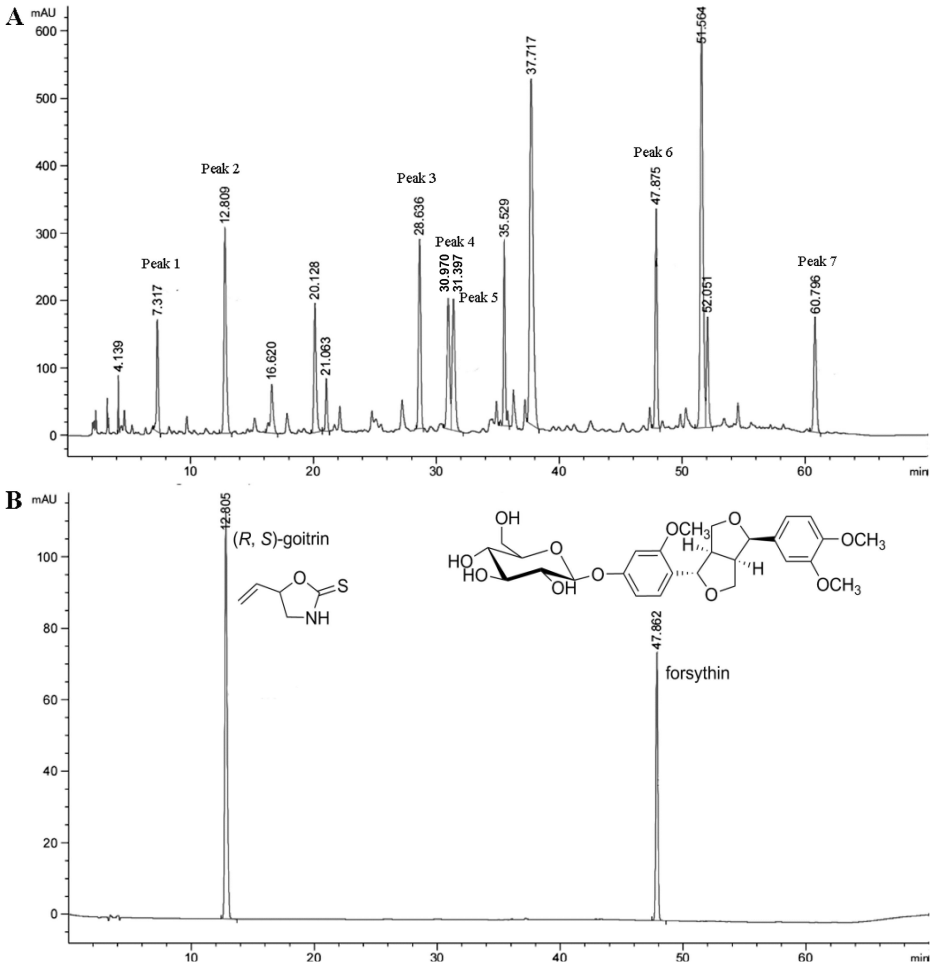

Figure 1 Characteristic map of AOL. (A) AOL. (B) Reference solution containing (R, S)-goitrin and forsythin, as well as their chemical structures. AOL, antiviral oral liquid.

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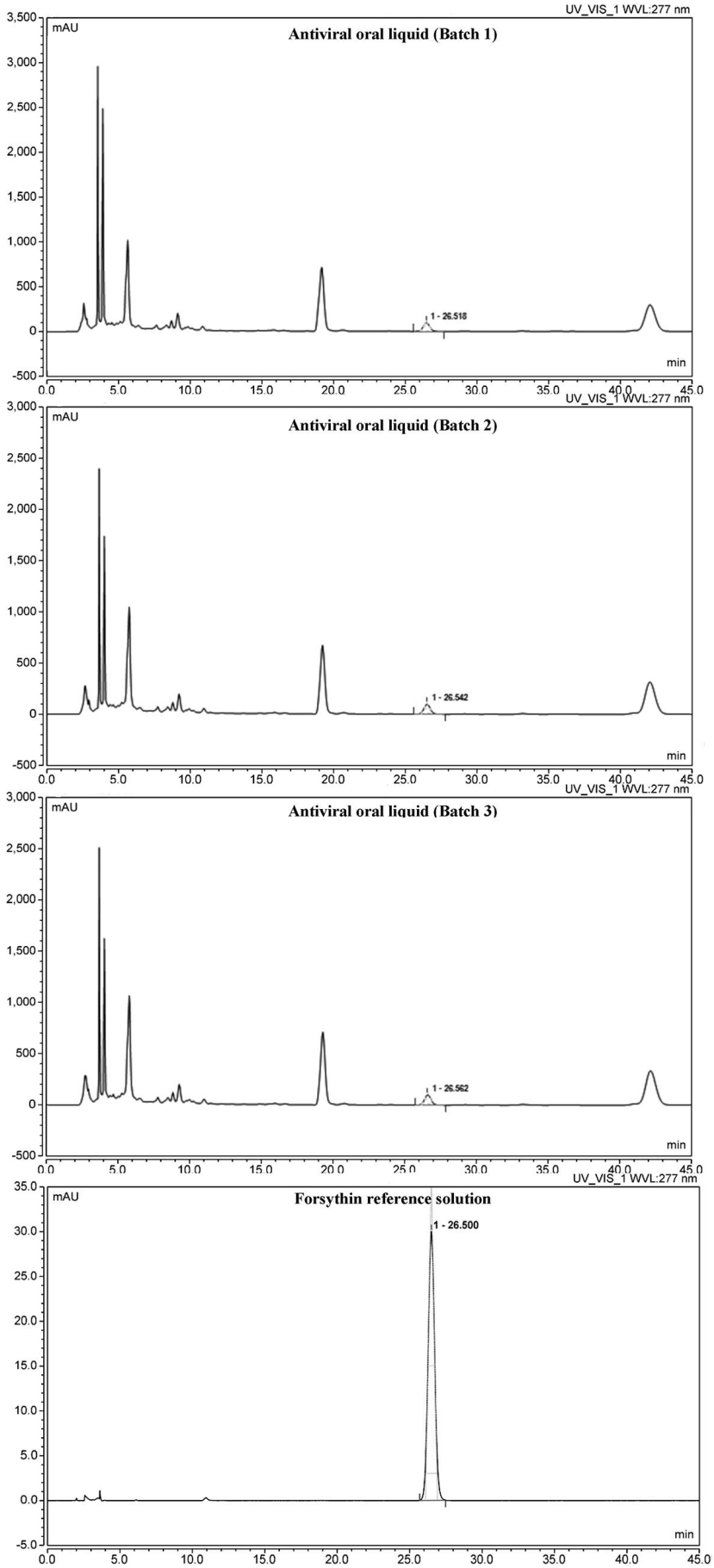

Figure 2 The forsythin content in AOL was determined by HPLC. AOL, antiviral oral liquid; HPLC, high-performance liquid chromatography.

Antiviral activity of AOL

Effects on mice model of influenza virus PR8-induced pneumonia. In this study, the experimental model of pneumonia induced by influenza virus PR8 was used to verify the antiviral effects of AOL at different doses. Lung index, the ratio of lung tissue weight to body weight indicates edema and inflammation in the lungs and is regarded as a classic indicator of antiviral activity [18]. As shown in Table 1, the lung index of mice infected with influenza virus PR8 was significantly higher than that of the normal control group ($P < 0.01$). AOL at 0.7 mL/kg/d dose significantly decreased the lung index of mice, and there was a significant difference compared with the model control group ($P < 0.05$).

Furthermore, hematoxylin and eosin staining was used to observe the effects of influenza virus and AOL on the pathological changes of lung tissue. As shown in Figure 3, the characteristics of lung injury caused by influenza virus intranasal drip in mice were as follows: loose swelling of pulmonary vessels and bronchus, increased exudate, collapse of alveolar wall, mild swelling, degeneration of alveolar

Table 1 Effect of AOL on pneumonia induced by influenza virus PR8 in mice ($\bar{x} \pm s$, n = 10)

Group (n = 10)	Lung index (g/100g)	Inhibition rate (%)
Normal	0.68 ± 0.07	---
Model	1.25 ± 0.12$^{\#\#}$	---
Oseltamivir	0.92 ± 0.12**	57.32
XFK oral liquid	1.16 ± 0.13	14.96
AOL (0.2 mL/kg/d)	1.26 ± 0.28	−2.41
AOL (0.5 mL/kg/d)	1.15 ± 0.13	17.95
AOL (0.7 mL/kg/d)	1.10 ± 0.17*	26.77
AOL (0.9 mL/kg/d)	1.13 ± 0.15	20.66
AOL (1.1 mL/kg/d)	1.23 ± 0.21	4.13
AOL (1.4 mL/kg/d)	1.25 ± 0.13	−0.20

$^{\#\#}P < 0.01$, compared with the normal group; $^{*}P < 0.05$, compared with the model group. AOL, antiviral oral liquid; XFK, Xiaoer Feire Kechuan.

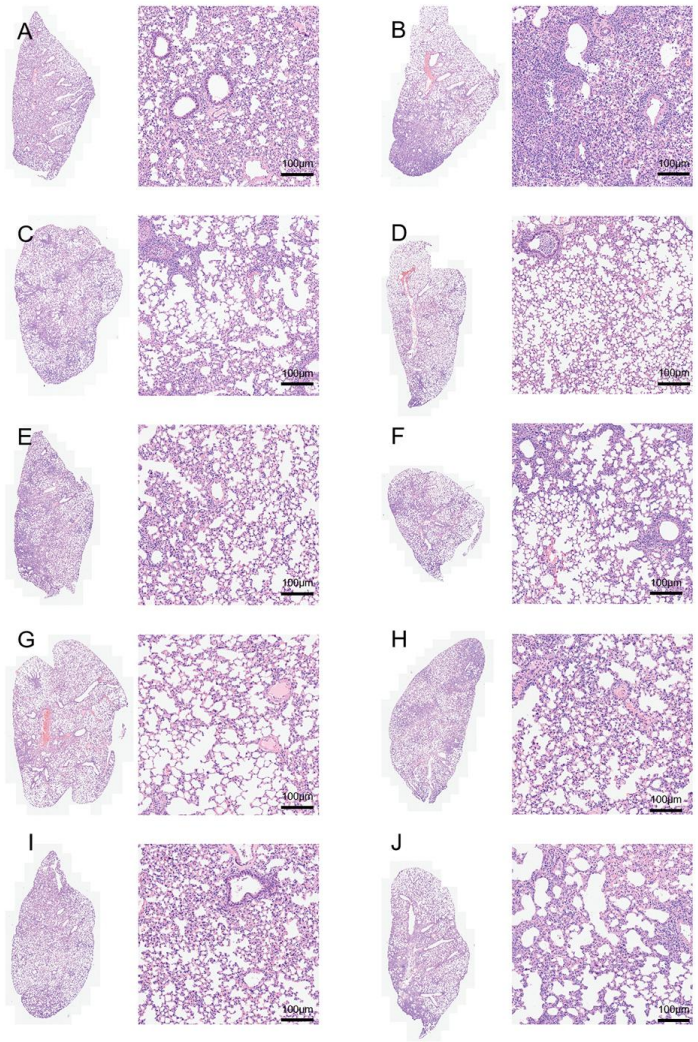

Figure 3 Pathological changes of lung tissue of mice induced by influenza virus PR8. (A) Normal, (B) Model, (C) Oseltamivir, (D) XFK oral liquid, (E) AOL at 0.2 mL/kg/d, (F) AOL at 0.5 mL/kg/d, (G)AOL at 0.7 mL/kg/d, (H) AOL at 0.9 mL/kg/d, (I) AOL at 1.1 mL/kg/d, (J) AOL at 1.4 mL/kg/d. AOL, antiviral oral liquid; XFK, Xiaoer Feire Kechuan.

epithelial cells and cell shedding. There were a large number of mononuclear cells, lymphocytes, a small number of neutrophil infiltration, interstitial cell proliferation. The bronchial epithelial lesions were serious, and most of the bronchial epithelial cells were denaturetic, necrotic, exfoliated or disappeared. The degree of pathological lesion in lung tissues showed a significantly decreasing trend by administration of AOL at 0.5, 0.7 and 0.9 mL/kg/d compared with the model group.

Inflammatory cytokines. In order to evaluate the effects of AOL on mice model of influenza virus PR8-induced pneumonia, we further performed ELISA to detect the expression of proinflammatory cytokines in lung. As is shown in Figure 4, the expression of IL-6 and TNF-α in mice infected with influenza virus PR8 was significantly higher than that of the normal control group ($P < 0.01$). The expression of IL-6 and TNF-α in lung showed a significantly decreasing trend by administration of AOL at 0.5, 0.7, and 0.9 mL/kg/d compared with the model group ($P < 0.01$).

Virus detection in lung tissue. In order to evaluate the anti-virus effects of AOL on the mice model of influenza virus PR8-induced pneumonia, we further performed RT-PCR to detect the viral load in lung tissue. As is shown in Figure 5, the PR8 expression in the model group was significantly higher than that of the normal control group ($P < 0.01$). The viral load showed a significantly decreasing trend by administration of AOL at 0.2, 0.5 and 0.7 mL/kg/d compared with the model group ($P < 0.01$).

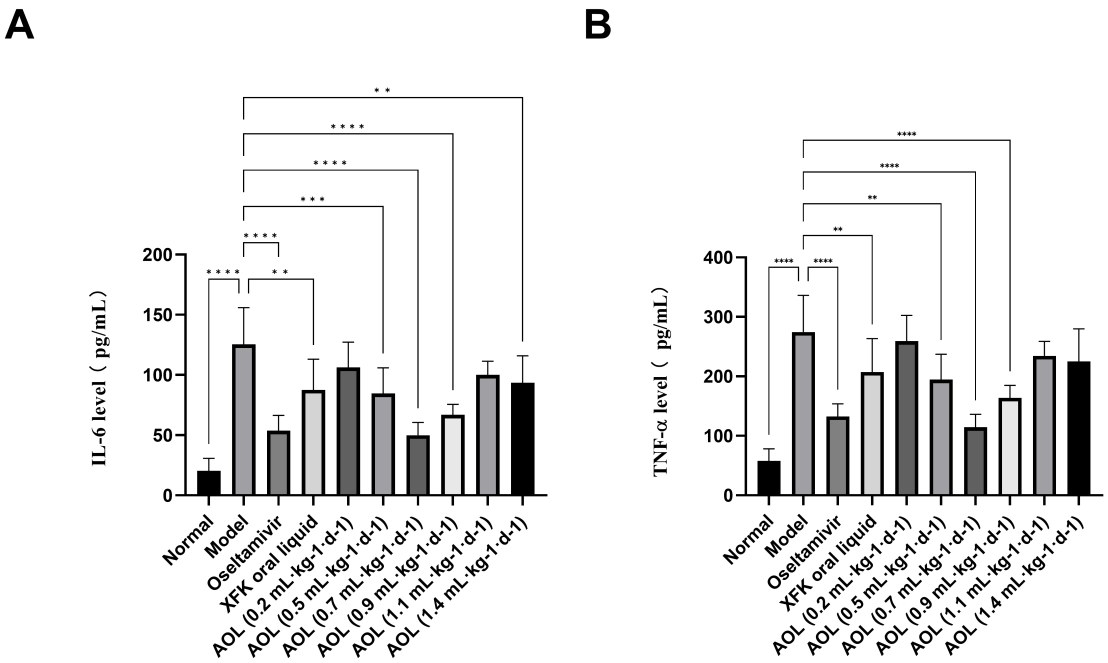

Figure 4 AOL inhibits inflammatory effects in PR8-induced pneumonia. The expression levels of (A) IL-6, (B) TNF-α. Data were presented as mean ± SD, n = 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, compared with the model group. AOL, antiviral oral liquid; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α.

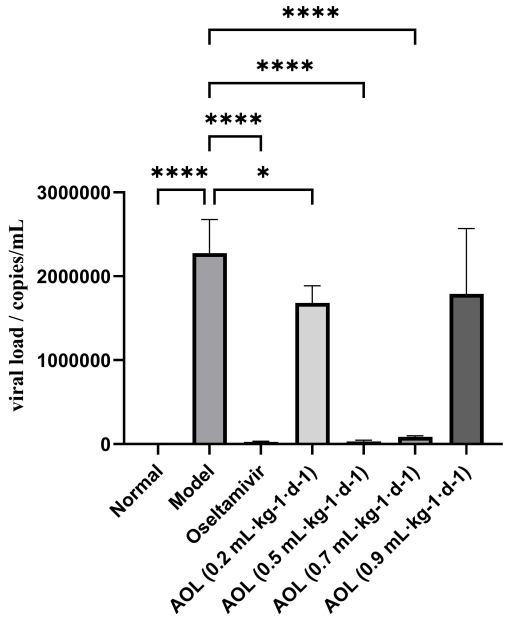

Figure 5 AOL inhibits viral load in PR8-induced pneumonia. The expression levels of viral load. Data were presented as mean ± SD, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, compared with the model group. AOL, antiviral oral liquid.

Protection against death in mouse model of influenza virus PR8-induced pneumonia. The mortality, average survival days, and life extension rate within two weeks after infection were calculated to evaluate the antiviral activity in this article. As shown in Table 2, the mortality rate of the mice infected with the influenza virus PR8 strain was 95%, and the average survival days was 8.00 ± 1.75 d. AOL at 0.5, 0.7, and 0.9 mL/kg/d lowered the mortality and prolonged the mean survival days compared with the model group ($P < 0.05$).

Anti-inflammatory activity of AOL

To clarify the anti-inflammatory activity of AOL, an acute inflammation mouse model of xylene-induced ear edema was established [14]. Ibuprofen was used as a positive drug in this study. The results were shown that AOL at a dosage range of 0.7–1.4 mL/kg/d significantly inhibited the ear edema, compared with the model group (Table 3). In addition, a mouse model with the increased permeability of abdominal capillaries induced by acetic acid was also used to verify the anti-inflammatory activity of AOL [17]. Intraperitoneal injection of acetic acid-induced acute inflammation in mice, resulting in increased capillary permeability in the abdominal cavity and increased exudation of Evans blue from intravascular to the abdominal fluid. Therefore, the absorbance value of peritoneal fluid at 590 nm was regarded as the indicator to evaluate the anti-inflammatory effect and measured by a microplate reader in this article. As shown in Table 3, AOL at 0.2–1.1 mL/kg/d could reduce the absorbance of mouse peritoneal fluid from 0.399 to 0.2, and the inhibition rate was up to 50%, which is similar to that of ibuprofen. These results suggested that AOL significantly inhibited the increase of abdominal capillary permeability induced by acetic acid.

Analgesic activity of AOL

Headaches and muscle aches are among the main symptoms of influenza, so the analgesic activity is also a critical efficacy indicator of influenza drugs. To investigate the analgesic activity of AOL, a writhing model induced by acetic acid was used in mice [18]. In short, 1% acetic acid was injected directly into the abdomen to stimulate the peritoneum and cause pain, resulting in writhing reaction in mice, which was manifested as abdominal concave, trunk and hind limb stretching, hip lifting, etc. As shown in Table 4, the writhing numbers of the mice treated with AOL at dosages of 0.7 and 0.9 mL/kg/d were significantly less than the model one. The results indicated that AOL exhibited a dramatic analgesic effect in the concentration of 0.7–0.9 mL/kg/d.

Antipyretic action of AOL

Fever is a common symptom of influenza. A lipopolysaccharide -induced fever in young rabbits was used to examine the antipyretic activity of AOL [17]. LPS is an endotoxin released by gram-negative bacilli, which can directly act on the thermoregulatory center of hypothalamus, or act on granulocyte or mononuclear macrophage to release leukocyte pyrogen. After leukocyte pyrogen enters the central nervous system, it can interact with the thermoregulatory center to up-regulate the temperature setting point, resulting in increased heat production, reduced heat loss, and elevated body temperature. Before the experiment, the young rabbits whose body temperature are 38.5–39.5 °C were selected. As shown in Table 5, the temperature of rabbits significantly increased 1–4 h after LPS administration. Compared with the model group, AOL at 0.7–1.1 mL/kg/d significantly reduced the body temperature at 2 and 3 h after drug

Table 2 Protective effects against death in mouse model of PR8-induced pneumonia ($\bar{x}$ ± s, n = 10)

Group (n = 20)	Number of deaths (n)	Mortality (%)	Death protection rate (%)	Mean survival days (d)	Life extension rate (%)
Model	19	95	--	8.00 ± 1.75	---
Oseltamivir	10	50	47.37**	11.90 ± 2.55**	48.75
XFK oral liquid	20	100	−5.26	8.45 ± 1.19	5.62
AOL (0.2 mL/kg/d)	19	95	0	8.60 ± 1.67	7.50
AOL (0.5 mL/kg/d)	15	75	21.05	9.80 ± 2.59*	22.50
AOL (0.7 mL/kg/d)	16	80	15.79	9.70 ± 2.52*	21.25
AOL (0.9 mL/kg/d)	18	90	5.26	9.35 ± 1.95*	16.88
AOL (1.1 mL/kg/d)	17	85	10.53	9.25 ± 2.40	15.63
AOL (1.4 mL/kg/d)	18	90	5.26	8.75 ± 2.20	9.38

*$P < 0.05$, **$P < 0.01$, compared with the model group. AOL, antiviral oral liquid; XFK, Xiaoer Feire Kechuan.

Table 3 The anti-inflammatory effect of AOL in the mouse model caused by dimethyl benzene and acetic acid ($\bar{x}$ ± s, n = 10)

Group (n = 10)	Ear edema (g)	Edema inhibition rate (%)	Absorbance (OD)
Normal	0.001 ± 0.004	---	0.084 ± 0.015
Model	0.014 ± 0.004##	---	0.399 ± 0.160##
Ibuprofen	0.006 ± 0.003**	55.56	0.165 ± 0.054**
XFK oral liquid	0.015 ± 0.007	−4.86	0.218 ± 0.072**
AOL (0.2 mL/kg/d)	0.011 ± 0.006	21.53	0.220 ± 0.092**
AOL (0.5 mL/kg/d)	0.013 ± 0.008	6.94	0.238 ± 0.054**
AOL (0.7 mL/kg/d)	0.007 ± 0.006*	50.00	0.176 ± 0.040**
AOL (0.9 mL/kg/d)	0.005 ± 0.002**	65.28	0.195 ± 0.046**
AOL (1.1 mL/kg/d)	0.006 ± 0.003**	55.56	0.229 ± 0.060**
AOL (1.4 mL/kg/d)	0.010 ± 0.004*	29.17	0.290 ± 0.143

##$P < 0.01$, compared with the normal control group; *$P < 0.05$, **$P < 0.01$, compared with the model group. AOL, antiviral oral liquid; XFK, Xiaoer Feire Kechuan.

Table 4 The analgesic effect of AOL in the mouse model of acetic acid-caused writhing ($\bar{x}$ ± s, n = 10)

Group (n = 10)	Numbers of writhing (n)
Normal	0 ± 0
Model	41.50 ± 13.04$^{\#\#}$
Ibuprofen	11.40 ± 9.26**
XFK oral liquid	24.70 ± 11.35**
AOL (0.2 mL/kg/d)	32.20 ± 11.07
AOL (0.5 mL/kg/d)	37.30 ± 11.15
AOL (0.7 mL/kg/d)	16.20 ± 11.32**
AOL (0.9 mL/kg/d)	18.20 ± 9.07**
AOL (1.1 mL/kg/d)	21.40 ± 12.46
AOL (1.4 mL/kg/d)	29.20 ± 9.80

$^{\#\#}P < 0.01$, compared with the normal control group; $^{**}P < 0.01$, compared with the model group. AOL, antiviral oral liquid; XFK, Xiaoer Feire Kechuan.

Table 5 Effect of AOL on the body temperature differences in the rabbit model of LPS-induced fever ($\bar{x}$ ± s, n = 6)

Group (n = 6)	ΔT (°C)			
	1 h	2 h	3 h	4 h
Normal	0.00 ± 0.15	0.03 ± 0.19	0.07 ± 0.14	0.03 ± 0.16
Model	1.58 ± 0.33$^{\#\#}$	2.03 ± 0.42$^{\#\#}$	1.88 ± 0.25$^{\#\#}$	1.07 ± 0.41$^{\#\#}$
Ibuprofen	1.00 ± 0.51*	0.88 ± 0.13**	0.60 ± 0.15**	0.27 ± 0.27**
XFK oral liquid	1.33 ± 0.10	1.75 ± 0.18	1.60 ± 0.19	0.88 ± 0.25
AOL (0.2 mL/kg/d)	1.28 ± 0.38	1.78 ± 0.20	1.65 ± 0.38	0.87 ± 0.21
AOL (0.5 mL/kg/d)	1.17 ± 0.38	1.67 ± 0.23	1.52 ± 0.41	0.95 ± 0.35
AOL (0.7 mL/kg/d)	1.27 ± 0.27	1.05 ± 0.21**	0.67 ± 0.18**	0.83 ± 0.29
AOL (0.9 mL/kg/d)	1.15 ± 0.37	1.23 ± 0.41**	1.23 ± 0.29**	0.95 ± 0.34
AOL (1.1 mL/kg/d)	1.32 ± 0.40	1.33 ± 0.48*	1.32 ± 0.35*	0.83 ± 0.27
AOL (1.4 mL/kg/d)	1.18 ± 0.21*	1.17 ± 0.36**	1.47 ± 0.19**	0.53 ± 0.33*

$^{\#\#}P < 0.01$, compared with the normal control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the model group. AOL, antiviral oral liquid; XFK, Xiaoer Feire Kechuan; LPS, lipopolysaccharide.

administration. A higher dose of AOL (1.4 mL/kg/d) considerably decreased the body temperatures for 1–4 h after drug administration.

Pharmacokinetics

The pharmacokinetic characteristics of AOL in mice were analyzed to elucidate its absorption and metabolism in the body. The main components of AOL including (*R*, *S*)-goitrin and forsythin could be detected in serum after administration for 1 h. But it was undetectable after administration for 12 h, which suggested that the half-life of AOL is less than 12 h. It provides a theoretical basis for the usage of AOL to be taken 3 times a day. In addition, the excretion of AOL was detected. As shown in Figure 6, forsythin was detected only in urine collected after administration. This shows that forsythin is mainly excreted through urine. While, (R, S)-goitrin can be detected in urine and feces collected after administration, most of it is in feces. These data indicated that the main components of AOL could be excreted from the body and the possibility of accumulation in the body causing toxicity is low.

Discussion

Influenza is a seasonal epidemic and pandemic caused by the influenza virus, a highly infectious virus [19]. Throughout the 19th and 20th centuries, it has caused millions of deaths worldwide. Thus, it is a serious global health problem. Influenza is one of the most common causes of respiratory illness in children with a high rate of morbidity and mortality [20]. There were about 870,000 children hospitalized due to influenza [21]. Therefore, it is of great importance to find new drugs for anti-influenza. Clinically, influenza is characterized by acute fever, chills, rhinorrhea, cough, sore throat, headache, and myalgias, so we performed a series of models to evaluate the efficacy and optimal dosage for children [22]. This study also provides references for the dose study in pediatrics.

Chinese medicines have been used for thousands of years to prevent and treat viral infectious diseases. Chinese medicine has unique advantages in treating influenza, and they have also accumulated a great deal of experience and research results in the prevention and treatment of influenza. Derived from the "*Treatise on Cold Damage Diseases*", AOL is widely used clinically for the prevention and treatment of hand, foot and mouth disease, colds, influenza virus infections, mumps and other diseases caused by viral infections.

In this study, we initially examined the optimal dosage of AOL by six young animal models that corresponded to the functional indications of AOL. According to its dosage for adults in clinical, we calculated and chose six dosages of AOL, including 0.2, 0.5, 0.7, 0.9, 1.1, 1.4 mL/kg/d for experiments. The results indicated that AOL could reduce the lung index and mortality, improve pathological changes and prolong the survival time of mice infected by the

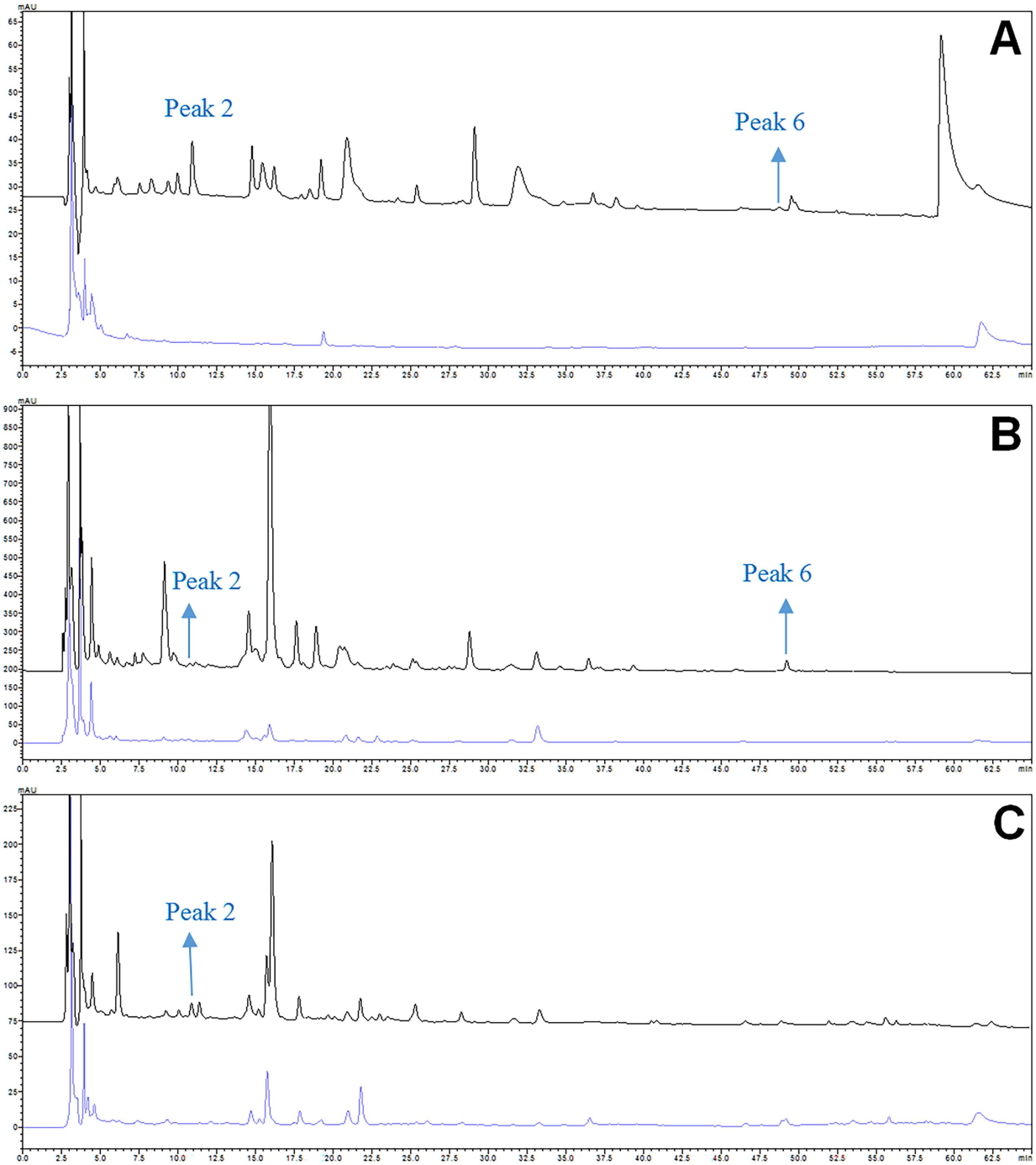

Figure 6 Pharmacokinetic analysis of AOL in vivo. HPLC analysis of serum samples (A) before (blue) and after (black) intravenous administration for 1 h, urine samples (B) and feces samples (C), before (blue) administration for 12 h and after administration for 24 h (black). AOL, antiviral oral liquid; HPLC, high-performance liquid chromatography.

Influenza A virus PR8 strains at 0.5–0.9 mL/kg/d. AOL at 0.7–1.4 mL/kg/d could significantly inhibit the ear shell swelling caused by xylene, and reduce the rabbit body temperature caused by LPS. As well as 0.2–1.1 mL/kg/d of AOL obviously inhibited mice capillary permeability of acute inflammation induced by glacial acetic acid. AOL at 0.7 and 0.9 mL/kg/d reduced the painful writhing times in young mice. The most effective doses for young mice and rabbits are 0.7 mL/kg/d. Based on these findings, the recommended dosage and usage for children of different ages could be obtained by further conversion. But considering that a significant difference might still exist between children and model animals and that the abilities of children of different ages to absorb, distribute, metabolize, and excrete drugs are different, more clinical trial data are needed to provide supporting evidence for the use of AOL in children in clinical practice.

In conclusion, at appropriate doses, AOL showed prominent antiviral, anti-inflammatory, analgesic, and antipyretic activities. Moreover, this work determined that 0.7 mL/kg/d is the optimal AOL dose for children. Although this requires more clinical data verification, it still provides experimental research for the clinical application of AOL in children.

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