Journal of Ethnopharmacology 155 (2014) 1575–1582

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

The roots of *Ilex asprella* extract lessens acute respiratory distress syndrome in mice induced by influenza virus

Wei-ping Dai [a,1], Geng Li [a,1], Xiong Li [a], Qiu-ping Hu [a], Jian-xing Liu [a], Feng-xue Zhang [b,*], Zi-ren Su [a], Xiao-ping Lai [c,**]

[a] Guangzhou University of Chinese Medicine, Guangzhou 510006, China
[b] Institute of Tropical Medicine, Guangzhou University of Chinese Medicine, Guangzhou 510405, China
[c] Dongguan Mathematical Engineering Academy of Chinese Medicine, Guangzhou University of Traditional Chinese Medicine, Dongguan 523808, China

ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form
7 June 2014
Accepted 25 July 2014
Available online 4 August 2014

Keywords:
Ilex asprella extract
Acute respiratory distress syndrome
Influenza virus
Immunoregulation
Viral load

ABSTRACT

Ethnopharmacological relevance: In traditional Chinese medicine, the root of *Ilex asprella* (Hook. & Arn.) Champ. ex Benth. (IA) has been widely used to treat influenza, lung abscess and other diseases in South China for many years. The present study is aimed at investigating the treatment effect of IA on acute respiratory distress syndrome (ARDS) induced by the H1N1 virus in mice.

Materials and methods: After being inoculated with several viral doses of influenza A/FM/1/47 H1N1 virus, mice were given oral administration of IA extract (500 mg/kg or 125 mg/kg per day) for five or 10 consecutive days, respectively. Mice survival rate and clinical condition were observed for 15 days after inoculation. Lung weight, pathological analysis and arterial blood gas analysis were assessed. Lung viral load was quantified by RT-PCR. Moreover, immunological analysis was measured by leukocyte counts and the levels of inflammatory cytokines, including IL-6, IL-10, TNF-α, IFN-γ, MCP-1 and IL-12p70 in serum of mice.

Results: We found that the extract of *Ilex asprella* at dosages of 500 mg/kg could effectively diminish mortality rate, and ameliorate lung edema and inflammation. Administration of IA extract significantly depressed the expression of IL-6, TNF-α and MCP-1, and significantly increased the expression of IL-10 and IFN-γ in serum. Simultaneously, the extract was also found to reduce the lung viral load and improve pulmonary ventilation.

Conclusion: The present study shows that the extract of IA has the potential to treat ARDS, due to its abilities of attenuation of systemic and pulmonary inflammatory responses and inhibition of viral replication.

1. Introduction

Respiratory virus infection rapidly progressing to severe respiratory failure and acute respiratory distress syndrome (ARDS) are the primary causes of death during an influenza pandemic (British Infection Society et al., 2007; Pham et al., 2013). Previous studies found that viral pneumonia complicated with ARDS results in 15–46% mortality (Jaber et al., 2010; Ríos et al., 2011). This fatal ARDS is a serious response to virulent viral infection and characterized by the development of noncardiogenic pulmonary edema and impaired gas exchange (Narula et al., 2010). Due to its high mortality, it has become a major global life-threatening and medically challenging issue.

As the theory Multiple organ dysfunction syndrome (MODS) has put forward, awareness of ARDS gradually turned to the study of inflammation reaction and its cytokines participated in the reaction have become a research hotspot (Bone, 1996a, 1996b). Studies have shown that the complex network of inflammation plays a vital role as signaling molecules that initiate, enhance, and perpetuate inflammatory responses on a local and systemic basis in the development of ARDS (Suter et al., 1992). Moreover, the determination of single cytokine in serum and bronchial alveolar lavage fluid (BALF) cannot accurately forecast the start or ending of ARDS. It is more meaningful to use pro-inflammatory and anti-inflammatory (pro- and anti-inflammatory) cytokines to assess the inflammatory reaction. Blocking inflammatory reaction has become the key to treat ARDS, and medicines that can balance inflammatory cytokines are urgently needed (Bone, 1996a, 1996b; Park et al., 2001).

* Corresponding author. Tel.: +86 20 3658 5148; fax: +86 20 3658 5148.
** Corresponding author. Tel.: +86 20 3935 8045; fax: +86 20 3935 8390.
E-mail addresses: zhangfengxue@gzucm.edu.cn (F.-x. Zhang), lxp88@gzhtcm.edu.cn (X.-p. Lai).
[1] These authors contributed equally to this work, and are the co-first writers.

http://dx.doi.org/10.1016/j.jep.2014.07.051
0378-8741/

The plant *Ilex asprella* (Hook. & Arn.) Champ. ex Benth., which is one of the native herbal medicines in South China, is mainly distributed in the Guangdong, Guangxi and Fujian provinces. It is popularly named "Gang-Mei" in folk; and its roots and leaves have been a common part of herbal tea in treating influenza, bacterial infections and lung abscess for hundreds of years (Zhou, 1999; Qi and Ren, 2009; Zeng et al., 2012). Previous experiments revealed that it mainly comprises triterpenoid saponin, phenolic acid and alkaloid phenolic compounds (Cai et al., 2010; Huang et al., 2012). It has been demonstrated that IA extract has significant anti-inflammatory and anti-influenza activity (Liu et al., 2004; Zhu et al., 2007; Luo et al., 2010). In earlier studies, we also found that IA water extract has inhibitory effects on Avian influenza virus H9N2 (Li et al., 2012).

There is limited pharmacological information about IA in treating ARDS caused by influenza virus. Therefore, this study was designed to elucidate its potential pharmacological mechanisms and consider whether IA extract could be used as a potential drug of H1N1-induced ARDS. To test our hypothesis, the mice model of ARDS induced by (A/FM/1/47 H1N1, FM1) virus was developed to study the therapeutic effect of IA. The survival rate, viral load in lungs, blood gas analysis, leukocyte counts, pro- and anti-inflammatory cytokines related to inflammatory response, and the histopathology of lungs in mice were addressed to show the effects.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

The kit of Trizol Reagent Invitrogen was purchased from Life Technologies Corporation (Carlsbad, USA). The kit of Influenza A Virus H1N1 type (IAV-H1N1) Fluorescence Polymerase Chain Reaction (PCR) Diagnostic was purchased from Vipotion Biotechnology Co. Ltd. (Guangzhou, China). The Wright–Giemsa stain was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The kit of BD™ Cytometric Bead Array (CBA) Mouse Inflammation was obtained from BD Biosciences (San Diego, USA).

2.1.2. Virus

A mouse-adapted (A/FM/1/47 H1N1, FM1) virus was kindly donated by the Institute of Tropical Medicine, Guangzhou University of Chinese Medicine. The virus was amplified in allantoic cavity of embryonated eggs for 48 h at 36 °C. The LD_{50} of virus in mice was determined by the Reed–Muench method ($LD_{50}=10^{-2.57}$/0.05 mL). The virus was stored at −80 °C. All tests were performed in class II biosafety safety cabinets.

2.1.3. Animals and influenza virus infection

Specific pathogen-free (SPF) KM mice of either sex, weighing between 13 g and 15 g, were obtained from the Laboratory Animal Services Center, Guangzhou University of Chinese Medicine (Guangzhou, China). The mice were housed in stainless steel cages with uniform temperature of 22 ± 2 °C, relative humidity of 50 ± 10%, 12 h/12 h light/dark cycles, and had food and water throughout all experiments. After being anesthetized with diethyl ether, mice were challenged intranasally with 50 μl (10 times the LD_{50} mouse infectious dose) of virus diluted in sterile saline. In the meantime, control group was challenged with equivalent amounts of PBS. An attemperator was used to keep mice warm after receiving anesthesia. The virus was kept on ice until the completion of the virus infection. All procedures and studies were conducted strictly in accordance with the National Institute of Health guidelines.

2.1.4. Plant and drugs

The roots of *Ilex asprella* (Hook. & Arn.) Champ. ex Benth. were collected on September 2012 from Pingyuan, Guangdong province, China. The plant was identified by Prof. Xiao-Ping Lai in the School of Chinese Materia Medica, Guangzhou University of Chinese Medicine. The roots were dried in shade. The voucher specimens (No. 20121103) were deposited in the herbarium of Guangzhou University of Chinese Medicine for further study.

Ribavirin tablets were purchased from Huiren Pharmaceutical Co., Ltd., Jiangxi, China (Standard: 100 mg/tablet, batch number: 1303010, product batch number: H20033538).

2.1.5. Preparation of the plant extract

The roots of *Ilex asprella* 1 kg were pulverized and extracted using 8 L 70% Ethanol for 2 h with a heating reflux method. The extract solution was filtered and concentrated using a rotary evaporator. The total phenolic acids and total saponins in IA extract were determined at 312 nm and 545 nm by a ultraviolet spectrophotometer, using Caffeic acid and 19-dehydrouraolic acid as the reference solution. The contents of total phenolic acids and total saponins were 0.20% and 3.45%, respectively. Then the extract was dried to obtain a powder on an atomizing drier. Finally, the extract was dissolved in distilled water before administration to animals during experiment.

2.2. Methods

2.2.1. Acute oral toxicity study

Six groups of mice ($n=10$) were used for the acute oral toxicity study. 5 groups were subjected to increasing IA extract doses (100 mg/kg, 500 mg/kg, 1000 mg/kg, 1500 mg/kg and 2000 mg/kg of body weight, respectively) by oral gavage while the control group was subjected to sterile water. Mice were monitored daily over 14 days for mortality, changes of behavioral and toxic effects. The living mice from each group were euthanized on day 15 to observe the pathological change of organs compared with the control group.

2.2.2. Drug administration

Five groups of mice ($n=22$) were used for the studies, 10 mice for survival study and 12 mice for the rest tests. The IA high (IAH) and light (IAL) mice were, respectively, orally administered with 500 mg/kg and 125 mg/kg of body weight per day IA extract. Ribavirin mice were orally administered with 7.5 mg/kg of body weight per day ribavirin as positive control to compare with IA extract. The remaining two groups (control and infected mice) received equivalent amounts of sterile water. Mice were administered like above mentioned 4–5 h after virus challenged for a different period of time. Mice in survival rate experiment were orally administered for 10 days and the rest of mice were administered for 5 days.

2.2.3. Survival rate

Ten mice from each group were monitored for 15 days post-infection. In addition, clinical signs and time of death were recorded daily.

2.2.4. Arterial blood gas analysis

Arterial blood gas analysis was operated as described by Fagan et al. (1999). Briefly, six mice from each group were anesthetized with intraperitoneal injection of chloral hydrate on day 6 p.i. Then, arterial samples (0.4 ml) were obtained from abdominal aorta with heparinized syringe from lightly anesthetized mice spontaneously breathing room air. Partial pressure of oxygen (PaO_2), partial pressure of carbon dioxide ($PaCO_2$), arterial oxygen saturation