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Glycyrrhizic acid as the antiviral component of *Glycyrrhiza uralensis* Fisch. against coxsackievirus A16 and enterovirus 71 of hand foot and mouth disease

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ABSTRACT

Ethnopharmacological relevance: The radices of *Glycyrrhiza uralensis* Fisch. and herbal preparations containing *Glycyrrhiza* spp. have been used for thousands of years as an herbal medicine for the treatment of viral induced cough, viral hepatitis, and viral skin diseases like ulcers in China. Glycyrrhizic acid (GA) is considered the principal component in *Glycyrrhiza* spp. with a wide spectrum of antiviral activity.

Aim: The present study attempt to validate the medicinal use of *Glycyrrhiza uralensis* for hand, foot and mouth disease (HFMD) and further to verify whether GA is an active antiviral component in the water extract of *Glycyrrhiza uralensis*.

Materials and methods: Radices of *Glycyrrhiza uralensis* Fisch. were extracted with hot water. The chemical contents of the extract were profiled with HPLC analysis. The antiviral activity of the extract and the major components was evaluated against infection of enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) on Vero cells. The cytopathic effect caused by the infection was measured with MTT assay. Infectious virion production was determined using secondary infection assays and viral protein expression by immunoblotting analysis.

Results: The extract at 1000 μg/ml suppressed EV71 replication by 1.0 log and CVA16 by 1.5 logs. The antiviral activity was associated with the content of GA in the extract since selective depletion of GA from the extract by acid precipitation resulted in loss of antiviral activity. In contrast, the acid precipitant retained antiviral activity. The precipitant at a concentration of 200 μg/ml inhibited EV71 and CVA16 replication by 1.7 and 2.2 logs, respectively. Furthermore, GA dose-dependently blocked viral replication of EV71 and CVA16. At 3 mM, GA reduced infectious CVA16 and EV71 production by 3.5 and 2.2 logs, respectively. At 5 mM, CVA16 production was reduced by 6.0 logs and EV71 by 4.0 logs. Both EV71 and CVA16 are members of Enterovirus genus, time-of-drug addition studies however showed that GA directly inactivated CVA16, while GA anti-EV71 effect was associated with an event(s) post virus cell entry.

Conclusions: This study validated the medicinal usefulness of radices *Glycyrrhiza uralensis* against the etiological agents of HFMD. In addition to the identification of GA as the antiviral component of *Glycyrrhiza uralensis* against EV71 and CVA16 infection, this study also reveals that GA inhibits EV71 and CVA16 with distinct mechanisms.

1. Introduction

Hand, foot and mouth disease (HFMD) is a common viral illness that usually affects infants and young children of 5 years old or younger. The clinical features of HFMD typically begin with mild fever, sore throat, poor appetite, and diarrhea. A day or two later, blisters form in the mouth and a rash develops on the cheeks, gums, and the tongue, potentially with life-threatening neurological

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complications, such as encephalitis. The causative agents of HFMD have been identified as coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) viruses, accounting for more than 70% of the cases during the 2008 outbreak in China (WHO, 2008). CVA16 and EV71 are single strand RNA viruses of the Enterovirus genus. The viruses can be spread through direct contact with blisters and other surfaces contaminated with virus-containing fluids or through fecal–oral route. HFMD occurs sporadically around the world since its first report in 1957. Large outbreaks of HFMD with higher morbidity and mortality have become common in the Asia Pacific region. An outbreak in 2008 affected nearly half million children with 126 deaths in eastern China (Yang et al., 2009). Data reported to the Ministry of Public Health of China showed that more than one million cases annually and hundreds of deaths associated with HFMD in China for the past 3 years (www.moh.gov.cn). There is no vaccine or specific antiviral drugs available for HFMD. Good hygiene, including hand washing and disinfection of surfaces in child care facilities, therefore remains as the most effective approach to reduce the transmission rate of HFMD (Solomon et al., 2010; Ma et al., 2011).

The radices of *Glycyrrhiza* spp., commonly known as "Gan Cao" in Chinese and licorice in English, have been used for thousands of years as an herbal medicine for the treatment of sore throat, cough, bronchitis, peptic ulcers (Das et al., 1989; Krausse et al., 2004), arthritis, and allergic diseases (Fiore et al., 2005; Asl and Hosseinzadeh, 2008; Kim et al., 2010). The plant extract has also been used as flavoring agents for food and beverages with a relative safe profile. During the outbreaks of HFMD in China, medicinal herbs or herbal preparations have demonstrated therapeutic efficacy by ameliorating the symptoms of the disease and/or shortening the course of the disease. Most of the herbs with reported therapeutic effectiveness have been used traditionally or folklorically for inflammatory and/or infective diseases (Xue et al., 2011; Cao et al., 2012).

Glycyrrhiza spp. have been reported with broad antiviral activity (Fiore et al., 2008) and glycyrrhizic acid (GA, also known as glycyrrhizin, glycyrrhizinic acid), the major bioactive component of *Glycyrrhiza* spp. has demonstrated activity against DNA and RNA viruses. GA at millimolar concentrations inhibits growth and cytopathology of several unrelated viruses, while not affecting cell activity and ability to replicate (Pompei et al., 1979). Subsequent studies have shown the antiviral activity of GA against several enveloped viruses, including HIV, and SARS related coronavirus (Cinatl et al., 2003; Hoever et al., 2005), Kaposi's Sarcoma-Associated herpesvirus (Curreli et al., 2005; Kang and Lieberman, 2011), hepatitis C virus (Ashfaq et al., 2011), respiratory syncytial virus, vaccinia virus, Epstein-Barr virus, and vesicular stomatitis virus (Fiore et al., 2005, 2008; Pompei et al., 2009). The compound has not previously been reported to inhibit the infection by nonenveloped viruses.

Some of the plants like *Glycyrrhiza uralensis* and *Houttuynia cordata* have been demonstrated with strong antiviral activity against EV71 infection (Kuo et al., 2009; Lin et al., 2009). However, the antiviral activity against CVA16 infection and the active components in those herbs are still undetermined. In this study, we aimed to assess the anti-EV71 and CVA16 infection activity of the water extract of *Glycyrrhiza uralensis*, and further determine whether antiviral activity of *Glycyrrhiza uralensis* is attributable to GA.

2. Materials and methods

2.1. Chemicals and antibodies

Antibodies to EV71 VP1 protein were purchased from Abnova (Taiwan), to human GAPDH from Bioworld Technology (Minneapolis, MN), and horse radish peroxidase (HRP) conjugated secondary antibodies from Sigma (St. Lois, MO). Chemicals including acyclovir (ACV), 18β-glycyrrhetinic acid (β–GA), ammonium glycyrrhizate, and methyl thiazolyl diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Liquiritin was from Chengdu Biopurify Pharmaceuticals (Chengdu). Chemiluminescent (ECL) reagent kit with enhanced detection sensitivity was purchased from Thermo Fisher (Pittsburgh, PA).

2.2. Cells and virus

African green monkey kidney Vero cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml of penicillin and streptomycin, and 2 mM L-glutamine.

CVA16 was kindly provided by Dr. C. Zheng of Wuhan University. EV71 was a clinical isolate of the C4 subgenotype by VP1 sequence analysis (Zhang et al., 2010). The viruses were propagated in Vero cells and virus titers were determined on Vero cells by measuring 50% tissue culture infective dose ($TCID_{50}$) for EV71 and by a plaque forming assay for CVA16.

2.3. Preparation of water extract of Glycyrrhiza uralensis

Slices of radices of *Glycyrrhiza uralensis* Fisch. were purchased from local drug stores in Nanjing and identified by Ms. Yunxia Xu (Research Assistant, Nanjing University) and with HPLC profiling following instructions in Chinese Pharmacopeia (Fig. 1). Voucher specimens (2011-18-1 to -5) were stored at Laboratory of Microbial Science and Pharmacy, School of Medicine, Nanjing University. The materials were ground and extracted with boiling water as described in Kuo et al. (2009). After adjusting the pH of the liquid (~6) with diluted NaOH, the liquid was filtered and tested for antiviral activities or lyophilized for future studies. The lyophilized powder was stored at −70 °C and reconstituted in de-ionized water (diH_2O) prior to experiments.

To deplete GA, the pH of extract was adjusted to pH 1.5 with 2 M H_2SO_4 to precipitate GA at 4 °C for overnight. The precipitant and the supernatant were collected by filtration. The precipitant was rinsed with cold water for two times and then lyophilized. Additionally, the supernatant was also lyophilized after adjusting pH with diluted NaOH. Both the precipitant and supernatant were reconstituted in diH_2O prior to experiments. Acid precipitation to concentrate glycyrrhizic acid from the water extract is effective (Lu et al., 2006) and is a standard procedure recommended by the US Food and Drug Administration (CFR Title 21, Sec. 184.1408 Licorice and licorice derivatives).

2.4. HPLC profiling and quantitative determination of the chemical components in the extracts

The identity of the water extract was characterized by HPLC profiling for glycyrrhizic acid and liquiritin as specified in Chinese Pharmacopeia. A BosChroma ODS-AQ column (4.6 × 150 mm, 5 mm particle size) and Acetonitrile–H_2O containing 0.1% trifluoroacetic acid (32:68) as a mobile phase were used. The components were monitored at 248 nm under a UV detector. Ammonium glycyrrhizate and liquiritin were used as standards for HPLC studies.

2.5. Infection assays

Vero cells in triplicate or duplicate were infected with EV71 or CVA16 virus at a multiplicity of infection (MOI) of 0.3. To test the inhibitory effect of a compound, a stock solution of a testing sample in PBS or in DMSO or solvent control (DMSO at 0.1%) was added to Vero cells for 2 h before infection. The compound was