Journal of Ethnopharmacology 148 (2013) 466–473

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Water extract of licorice had anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines

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ARTICLE INFO

Article history:
Received 3 December 2012
Received in revised form
11 April 2013
Accepted 18 April 2013
Available online 30 April 2013

Keywords:
Glycyrrhiza uralensis
Respiratory tract infection
RSV

ABSTRACT

Ethnopharmacological relevance: Licorice (*Glycyrrhiza uralensis* Fisch., Leguminosae) has been used in herbal medicine and food supplement worldwide for centuries. Licorice is a common ingredient of several prescriptions of traditional Chinese medicine which have been proved to inhibit infection of human respiratory syncytial virus (HRSV). There are two preparations of licorice, Radix Glycyrrhizae and Radix Glycyrrhizae Preparata. However, it is unknown whether licorice or which preparation of licorice is effective against HRSV, nor is its active constituent.

Aim of the study: We tested the hypothesis that Radix Glycyrrhizae can effectively decrease HRSV-induced plaque formation in respiratory mucosal cell lines. We also tried to find out the active constituent.

Materials and methods: Anti-HRSV activities of hot water extracts of preparations of licorice, glycyrrhizin and 18β-glycyrrhetinic acid (18β-GA), the active constituents of licorice, were examined by plaque reduction assay in both human upper (HEp-2) and low (A549) respiratory tract cell lines. Abilities of crude licorice to inhibit viral replication and to stimulate IFN-β were evaluated by reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively.

Results: Radix Glycyrrhizae and Radix Glycyrrhizae Preparata dose-dependently inhibited HRSV-induced plaque formation in both HEp-2 and A549 cell lines ($p < 0.0001$). The effect of Radix Glycyrrhizae was better than that of Radix Glycyrrhizae Preparata on HEp-2 cells. However, there was no difference of their anti-HRSV effects on A549 cells. Besides, glycyrrhizin was ineffective at all. Nevertheless, 18β-GA showed a potent anti-HRSV activity. Radix Glycyrrhizae was more effective when given before viral inoculation ($p < 0.0001$) which may be due to its inhibition of viral attachment on ($p < 0.0001$) and penetration ($p < 0.0001$) into the host cells. The anti-HRSV activity of Radix Glycyrrhizae was further confirmed by RT-PCR and qRT-PCR. 300 μg/ml Radix Glycyrrhizae markedly decreased the viral amounts within the cells and in the suspension. Radix Glycyrrhizae might further stimulate mucosal cells to secrete IFN-β to counteract viral infection.

Conclusions: Both Radix Glycyrrhizae and Radix Glycyrrhizae Preparata are effective against HRSV infection on airway epithelial cells. Radix Glycyrrhizae inhibited HRSV mainly by preventing viral attachment, internalization, and by stimulating IFN secretion. 18β-GA may be one of its active constituents.

1. Introduction

Viral Bronchiolitis and pneumonia are the common low respiratory tract infection in infants and children under 2 years of age. Bronchiolitis could cause respiratory failure in infants and children (Abboud et al., 2012; Jat and Chawla, 2012). Human respiratory syncytial virus (HRSV), rather than the influenza A virus, is the most important cause of viral bronchiolitis (Macao

Abbreviations: 18β-GA, 18β-Glycyrrhetinic acid; A549, human lung carcinoma cell; ATCC, the American Type Culture Collection; CC_{50}, 50% cytotoxic concentration; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FDA, Food and Drug Administration; HEp-2, Human larynx epidermoid carcinoma cell; IC_{50}, minimal concentration required to inhibit 50% cytopathic effect; IFN, interferon; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; pfu, plaque forming unit; HRSV, Human respiratory syncytial virus.

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http://dx.doi.org/10.1016/j.jep.2013.04.040

et al., 2011). Furthermore, HRSV is the major pathogen of viral pneumonia in children (Falade and Ayede, 2011). The incidence of HRSV-induced pneumonia is six times more than that of influenza A virus in children under 5 years of age (Hatipoglu et al., 2011). It has been estimated that more than 1.5 million children younger than 5 years old annually died of pneumonia worldwide (Maxwell et al., 2012). Therefore, HRSV is the most common cause of hospitalization due to severe acute low respiratory infection in infant and children (Nair et al., 2010). Ribavirin is an FDA-approved agent to manage HRSV infection. However, Ribavirin, with continuous inhalation for 20 h to get the therapeutic effect, is inconvenient to use (Shigeta, 2000). Application of ribavirin is further limited by its side effects (De Franceschi et al., 2000; Empey et al., 2010). Therefore, the American Academy of Pediatrics does not recommend ribavirin to manage HRSV infection (Empey et al., 2010). Palivizumab, a humanized monoclonal IgG antibody against HRSV, effectively prevents HRSV infection. Nevertheless, it is expensive and ineffective to manage an established HRSV infection (Shadman and Wald, 2011). Development of new anti-HRSV chemotherapies is urgently needed.

Licorice (*Glycyrrhiza uralensis* Fisch.; Leguminosae) is a common ingredient in prescriptions of traditional Chinese medicine. Ge-Gen-Tang (GGT; kakkon-to) (Chang et al., 2012a), Liu-He-Tang (LHT) (Chang et al., 2011), Sheng-Ma-Ge-Gen-Tang (SMGGT; Shoma-kakkon-to) (Wang et al., 2011b) are all Chinese traditional prescriptions for airway symptoms that have been proved to have anti-HRSV activity. These prescriptions contain *Glycyrrhiza uralensis*. Therefore, we hypothesized that *Glycyrrhiza uralensis* might be one of the active ingredients that accounts for their anti-HRSV activity. However, *Glycyrrhiza uralensis* is also the most common ingredient in numerous Chinese traditional prescriptions. Therefore, it might be a bystander that is co-incidentally present within these prescriptions. Besides, there are two preparations of Chinese licorice. One is crude licorice, Radix Glycyrrhizae, and the other is honey-burn processed licorice, Radix Glycyrrhizae Preparata (Radix Glycyrrhizae Praeparata cum melle). Radix Glycyrrhizae has been used to manage various symptoms in different organ systems worldwide, including sore throat and infectious diseases (Fiore et al., 2008; Shi et al., 2012). Extracts of Radix Glycyrrhizae and its active constituents have been reported to have anti-cancer activity (Seon et al., 2012), organ-protective effect (Kim et al., 2006; Zhang et al., 2011), protection from heavy metal injury (Kim et al., 2008), anti-oxidative and anti-inflammatory activity (Chin et al., 2007; Wu et al., 2011). In vitro antiviral activities of Radix Glycyrrhizae have been reported against human immunodeficiency virus (Sasaki et al., 2002), Japanese encephalitis virus (Badam, 1997), EV71 (Kuo et al., 2009), Rotavirus (Kwon et al., 2010), SARS-associated coronavirus (Cinatl et al., 2003), Epstein–Barr virus (Lin, 2003), and Flaviviruses (Crance et al., 2003). Its antiviral activity against HRSV has been reported in HeLa cells (Dong et al., 2004; Wang et al., 2006). However, HeLa cells are not respiratory tract cells. The actual anti-HRSV activity of Radix Glycyrrhizae has been questioned. In contrast, Radix Glycyrrhizae Preparata is commonly used to manage weakness and palpitation. However, study of Radix Glycyrrhizae Preparata is rare and its bioactivity is not fully understood. Bioactivity of a crude medicinal plant can be changed during preparation. For example, dried ginger is prepared from fresh ginger and loose its anti-HRSV activity during processing (Chang et al., 2012a). Therefore, it was interesting to know whether Radix Glycyrrhizae or Radix Glycyrrhizae Preparata has antiviral activity against HRSV. We used both human upper (HEp-2) and low (A549) respiratory tract cell lines to test the hypothesis that Radix Glycyrrhizae, but not Radix Glycyrrhizae Preparata, could effectively inhibit plaque formation induced by HRSV infection. We also examined the anti-HRSV of its active constituents.

2. Materials and methods

2.1. Preparation of hot water extracts of licorices

Air-dried root of *Glycyrrhiza uralensis* was collected from markets in South Taiwan. A voucher specimen was prepared and deposited at Kaohsiung Medical University (KMU) Herbarium. The authenticity had been examined by experts at least twice through morphological and anatomical identifications. Furthermore, the reverse-performance liquid chromatography was used to establish its HPLC fingerprint. This confirmation was performed on a Hyperclone ODS C_{18} column (4.6×250, 5 μm). Acetonitrile-0.1% phosphoric acid was selected as the mobile phase gradient elution. The HPLC fingerprint data showed the standard glycyrrhizin ammonium salt, retention time nearby 48 min, was one of the major constituents of licorice (Fig. S1). Our fingerprint was similar to that of the root of *Glycyrrhiza uralensis* Fisch. (Wang and Yang, 2007). Furthermore, the content of liquiritin, retention time nearby 22 min, has been shown to be much less than those of glycyrrhizin in *Glycyrrhiza glabra* and *Glycyrrhiza inflate* (Rauchensteiner et al., 2005). The HPLC fingerprint data (Fig. S1) further confirm the air-dried root we used is not *Glycyrrhiza glabra*, nor *G. inflate*.

A hot water extract of crude licorice, Radix Glycyrrhizae, was prepared as reported before (Chang et al., 2012b) with the weight/weight (w/w) yield of 27.6%. Radix Glycyrrhizae Preparata was prepared by slicing crude licorice, mixed well with honey, and heated on a small fire until the color turn golden or deep yellow. Its hot water extract was prepared as above with the w/w yield of 36.6%. The extracts of licorices were dissolved in Dulbecco's modified Eagle's medium (DMEM)(Gibco BRL, NY) supplemented with 2% or 10% fetal calf serum (FCS) into the final concentrations (10, 30, 100, 300 μg/ml for bioactivity assay and up to 3000 μg/ml for cytotoxicity test) before experiments.

2.2. Cells, virus, and reagents

Human airway mucosal cell lines, HEp-2 (human larynx epidermoid carcinoma cells; ATCC CCL 23) and A549 cells (human lung carcinoma cells; ATCC CCL-185), were used to inoculate human respiratory syncytial virus (HRSV Long strain: ATCC VR-26). Cells were propagated at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. 2% FCS, instead of 10%, was used to culture virus-infected cell monolayer. Glycyrrhizin (Sigma, MO), the main active constituent of *Glycyrrhiza uralensis,* and 18β-glycyrrhetinic acid (18β-GA; Sigma, MO), an active constituent of licorice and also the primary metabolite of glycyrrhizin, were also tested. Virus was stored at −80 °C and its titer was determined by plaque assays expressing as plaque forming units per ml (pfu/ml).

2.3. Cytotoxicity assay

Cytotoxicities of glycyrrhizin, 18β-GA, and different preparations of licorice on HEp-2 and A549 cells were assayed by XTT-based method (Wang et al., 2011b). Briefly, 1×10^4/well cells were seeded into 96-well culture plates and incubated overnight at 37 °C under 5% CO_2. Then, culture medium was replaced by different concentrations (10, 30, 100, 300, 1000, 3000 μg/ml) of preparations of licorice in triplicate. After 3 days of incubation, their cytotoxicity were determined by XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid) kits (Roche, Germany). Their 50% cytotoxic concentrations (CC_{50}) were calculated by regression analysis of the dose–response curve generated from the data.