Antiviral Activity of GuiQi Polysaccharides against Enterovirus 71 in vitro

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In this study, we have investigated the antiviral activity of GuiQi polysaccharides (GQP) upon enterovirus 71 (EV71) in vitro. An assay using methyl thiazolyl tetrazolium (MTT), and analyses of cytopathic effects (CPE) were used to examine the antiviral activity of GQP upon Vero cells infected with EV71. The results revealed that GQP at concentrations below 31.2 μg/mL exhibited significant antiviral effects upon EV71 when applied under three different experimental protocols. GQP was most strongly active in preventing the adsorption of EV71 to target cells and in this respect it was significantly more effective than ribavirin. In addition, it was clear that GQP could inhibit viral replication when added to cells 2 h after infection, but if added at the point of infection its effect was weak. GQP is considered to be less toxic than ribavirin, and may warrant further evaluation as a possible agent in the treatment of hand, foot and mouth disease (HFMD).

Enterovirus 71(EV71); GuiQi polysaccharides (GQP); Vero cells; Antiviral activity; In vitro

INTRODUCTION

Enterovirus 71 (EV71) is an important human pathogen that may cause severe neurological complications and death in children under 6 years old (Chen S P, et al., 2010). There have been several outbreaks in the Asia-Pacific region during the past two decades and the virus has been considered a significant public health problem in the post-poliovirus eradication era (Solomon T, et al., 2010). In contrast to poliovirus, there are no effective vaccines or approved antiviral drugs available for use against EV71 (Wu K X, et al., 2010).

Supportive therapy is still the primary management strategy in severe cases of EV71 infection (Schezenmeir J D, et al., 2001; Vasiljevic T, et al., 2007). In addition to symptomatic treatment, intravenous immunoglobulin (IVIG) is used clinically to neutralize the virus and to suppress inflammation nonspecifically. Nevertheless, although this treatment has been applied routinely in severe cases of EV71 infection, its efficacy requires further evaluation (Nolan M A, et al., 2003). Therefore, the development of specific antiviral strategies against EV71 has become an urgent issue for the protection of children from the hazards of EV71 infection.

Plant polysaccharides are often identified as biological response modifiers (BRMs). Recently, greatly increased attention has been paid to plant polysaccharides, on account of their wide range of biological activities and their potential as sources of new ‘functional foods’, healthcare products and medicines (Chen L R, et al., 2009; Qiao D L, et al., 2009; Sun Y X, et al., 2008; Sun Y X, et al., 2009).

Angelica sinensis (Oliv.) Diels (Umbelliferae), known as Dang-gui in Chinese and used widely as a tonic agent, is one of the most important traditional Chinese herbs (Hsu H Y, et al., 1976) and polysaccharides are amongst its principal pharmacologically active constituents. The pharmacological properties of A. sinensis include anti-oxidative, anti-inflammatory, and immunomodulatory activities (Cho C H., et al. 2000; Mei Q B, et al., 1991).

The roots of Astragalus membranaceus (AM) are also amongst the most popular health-promoting herbs in China and their use dates back more than 2000 years.
Modern pharmacological studies have shown that the root of *A. membranaceus* possesses therapeutic activity as an immunostimulant, a tonic (adaptogenic), a hepatoprotective, a diuretic and an anti-diabetic agent (Ma X Q, et al., 2002). In addition, clinical studies have indicated that AM polysaccharides can counteract the side effects of chemotherapeutic drugs, notably by causing a substantial reduction in the degree of myelosuppression in cancer patients (Tin M, et al., 2007).

Many studies have indicated that a combination of the polysaccharides from *Angelica* and *Astragalus* roots, Guiqi polysaccharide (GQP), can exhibit – very likely synergistically in relation to its components – a range of antioxidant, anti-inflammatory and immunostimulatory effects. However, little research has yet been undertaken to characterize the pharmacology of GQP at a fundamental level and to relate it to the extraction methods that are used in the production of this traditional Chinese medicine.

The purpose of the present work was to extract and purify GQP and to examine whether GQP has anti-EV71 activity *in vitro*. For this purpose, cell survival, inhibition rates and cellular morphology were used as indices to evaluate the anti-EV71 activity of GQP by methyl thiazolyl tetrazolium (MTT) and observations of cytopathic effects (CPE). To our knowledge, this is likely to be the first report of the anti-EV71 activity of GQP *in vitro*.

**MATERIALS AND METHODS**

**Medicinal herbs and reagents**

*A. sinensis* and *A. membranaceus* were purchased from Minxian Shunfa Medicinal Material Co. (Gansu Minxian City, China). Dimethyl sulfoxide (DMSO) and MTT were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Auckland, New Zealand). Cell plates (96-well) were obtained from Corning Incorporated (New York, USA). Ribavirin (110502) was purchased from Tianjin Pharmaceutical Group Xinzheng Co., Ltd, Henan, China. All other reagents were of analytical grade and commercially available.

**Enterovirus 71 and Vero cell line**

EV71 was donated by the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Science (Guangzhou, China) and was propagated in African green monkey kidney (Vero) cells at 37 °C.

Vero cells were obtained from the Central Laboratory of the School of Life Science and Engineering, Lanzhou University of Technology, Lanzhou, China. They were grown in DMEM, supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) fetal calf serum (FCS). FCS was reduced to 2% (v/v) for the viral infection. Titer of the virus was quantified on Vero cell monolayers by determination of the 50% Tissue Culture Infective Dose (TCID₅₀), according to the Reed-Muench method (Yin Z, et al., 1997). The TCID₅₀ value for EV71 was assayed to be 10⁻⁴.⁶/0.1 mL. A virus solution of 100× the TCID₅₀ was used for the experiment.

**Cell seeding and infection of cell cultures**

Vero cells were seeded into 96-well plates, at 3×10³ cells/well, and incubated at 37 °C in an atmosphere of 5% CO₂ until a cell monolayer had been formed. The cell monolayer was then washed thoroughly twice with phosphate buffered-saline (PBS, pH 7.4), the reagents or the virus were added, and the results were quantified. In this antiviral experiment, cells treated with the agents served as a cell control group, cells infected with the virus acted as a virus control group, and cells infected with the virus plus ribavirin served as a positive control.

**Isolation and purification of Guiqi polysaccharide (GQP)**

A sliced mixture of untreated *Angelica* and *Astragalus* roots (in the ratio of 1:5, by weight) was extracted by refluxing twice with 95% ethanol, for 2 h each time, to remove pigments and small lipophilic molecules. The residue was then extracted with distilled water (in the ratio of 1:10, w/v) three times at 90 °C, for 3 h each time. All the aqueous extracts were then combined, filtered, concentrated, and precipitated with anhydrous ethanol (extract:ethanol, 1:4, v/v) at 4 °C for 24 h, in order to precipitate the polysaccharides. Following centrifugation at 4000 g for 20 min, the collected precipitate was first de-proteinated by the Sevag method (Sun Y X, et al., 2008) and then washed, in turn, with 95 % ethanol, 100 % ethanol, and acetone, before being dried to yield a crude preparation of GQPs. The crude preparation was re-dissolved in distilled water and applied to a DEAE-52 cellulose column, equilibrated with distilled water. The polysaccharides were fractionated and eluted with distilled water followed by a step gradient of NaCl (0.05, 0.1, 0.25 and 0.5 M ). The eluted polysaccharide fractions were then concentrated, before being further fractionated by size-exclusion chromatography on a Sephadex G-100 column and eluted with distilled water at a flow rate of 0.5 ml/min. The fractions were assayed spectrophotometrically by the phenol-sulfuric acid method (Dubois M, et al., 1956).