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***In vitro* anti-viral effect of fructopyrano-(1→4)-glucopyranose from *Radix isatidis* on influenza virus A**

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Abstract

To investigate the *in vitro* anti-viral effect of fructopyrano-(1→4)-glucopyranose (FG) from traditional Chinese medicine *Radix isatidis*, dog kidney cells (MDCK) were with treated with either FG and then with virus (Group A); virus and then with FG (Group B); or FG and virus simultaneously (Group C). Results showed the *in vitro* cell protection rate and virus inhibition rate increased significantly with the increase of FG concentration when compared with cells without FG treatment ($p < 0.01$). The anti-viral effect was the best in Group C and that in Group A was superior to that in Group B. FG could exert significant inhibited effect on neuraminidase activity with the IC_{50} of 0.1 mmol/mL and on hemagglutinin of avian influenza virus with minimum inhibitory concentration of 3.1 mg/mL. Our findings indicate FG can confer obvious anti-viral effects on influenza A virus *in vitro*.

Introduction

Radix isatidis (Banlangen in Chinese), dried roots of *Isatis indigotica* Fort, is a valuable Chinese materia medica (CMM) with antibacterial and anti-viral activities, improving immune function, anti-tumor effect, etc. Clinically, it is applied in the treatment of infections, especially viral infections. With the wide application of natural medicine, the pharmacological activities of *R. isatidis* and its active ingredients have been studied in-depth. Among the numerous active ingredients, fructopyrano-(1→4)-glucopyranose (FG) is a compound with potent immune activity and abundant in *R. isatidis*. FG was firstly isolated and purified from *R. isatidis* by our group and identified as a new disaccharide compound by NMR. Currently, there is no Chinese name for it and the structural formula is shown in Figure 1. FG is colorless and transparent with purity of >99% and molecular weight of 342. Preliminary study showed the anti-viral activity of FG *in vitro*. In the present study, the anti-viral activity of FG was evaluated by using a

variety of measures.

Materials and Methods

Virus

The influenza virus (A/PR8/34) was frozen in the

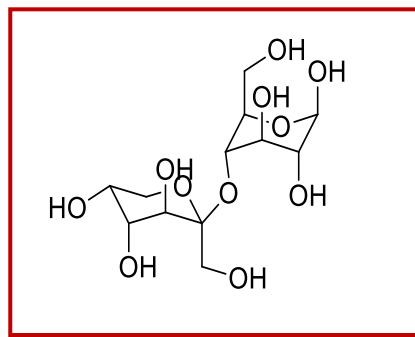


Figure 1: Chemical structural formula of FG



Academy of Military Medical Institute of Infectious Diseases and Microbiology.

Cell culture

Dog kidney cells (MDCK) were purchased from the Academy of Military Medical Institute of Infectious Diseases Microbiology and maintained in Eagle's MEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, kanamycin and glutamine (pH 7.0~7.2) in an atmosphere with 5% CO₂ at 37°C. Passaging was performed twice weekly.

Drugs and reagents

Ribavirin (Lot 110308) were purchased from Sichuan Medco Pharmaceutical and peramivir from Yuancheng Pharmaceutical & Chemical Co. Ltd. Fetal and bovine serum albumin (Gibco Biotech Co., Ltd), tetramethyl thiazolyl blue (MTT), dimethyl sulfoxide, NP-40, 2-(N-morpholino) ethanesulfonic acid (MES) and substrate (MUNANA) (Sigma), receptor destroying enzyme (RDE) (H-Y Biotech Co., Ltd) were used in the present study.

Detection of cytotoxicity

MDCK cells were seeded into 96-well plate (100 µL/well; 1 × 10⁴ cells/well). When cell confluence reached about 100%, the medium was removed and then treated with FG at different concentrations (starting at 100 mg/mL; 200 µL/well) and 4-wells were included in each group. Incubation was performed in an atmosphere with 5% CO₂ for 48 hours at 37°C. The cell morphology was observed daily under an inverted microscope and the cytopathic effect (CPE) on MDCK cells was determined. Then MTT assay was performed. In brief, 20 µL of MTT solution (5 mg/mL) was added to each well followed by incubation in an atmosphere with 5% CO₂ for 4 hours at 37°C followed by addition of 150 µL of dimethyl sulfoxide to each well and incubation under continuously shaking for 10 min. The optical density was measured at 490 nm on a microplate reader. The inhibition rate of cell growth was calculated as follow: IR = (OD_{control} - OD_{treatment})/OD_{control} × 100%. The maximum non-toxic concentration (TC₀) was also determined (Mosmann, 1983). Experiment was performed in triplicate and averaging was performed.

Determination of anti-viral effect

MTT assay was performed to determine the anti-viral effect of FG in three groups according to previously described (Knox et al., 2003; Oosterheert et al., 2005).

1) Group A: MDCK cells were seeded into a 96-well plate and treated with FG at four different concentrations (the lowest concentration was TC₀). In the positive control group, Ribavirin was added. Incubation was performed at 37°C for 2 hours. Following washing in PBS twice, 100 TCID₅₀ of influenza virus was added.

In the blank control, none was added, and virus was added in the absence of FG in the virus control groups. Incubation was done in an atmosphere with 5% CO₂ for 2 hours at 37°C and then the medium was refreshed with drug-free medium followed by incubation in an atmosphere with 5% CO₂ for 24 hours at 37°C. Optical density was measured as described in MTT assay above.

2) Group B: MDCK cells seeded into 96-well plate and then treated with 100 TCID₅₀ of influenza virus followed by incubation in an atmosphere with 5% CO₂ for 2 hours at 37°C. After washing in PBS twice, FG at four concentrations above was added with the lowest concentration of TC₀. In the blank control group, no treatment was performed and in virus control group, only virus was added. In the positive control group, peramivir was added. Incubation was performed in an atmosphere with 5% CO₂ for 2 hours at 37°C. Then, the medium was refreshed with drug-free medium followed by incubation in an atmosphere with 5% CO₂ for 24 hours at 37°C and optical density was measured as described in MTT assay above.

3) Group C: Cells were independently mixed with FG at four concentrations with the lowest concentration of TC₀. Blank control group and virus control group were also included. In the positive control group, peramivir was added. Incubation was performed in an atmosphere with 5% CO₂ for 2 hours at 37°C. Then, the medium was refreshed with drug-free medium followed by incubation in an atmosphere with 5% CO₂ for 24 hours at 37°C and optical density was measured as described in MTT assay above.

The cell protection rate (CPR) of FG on MDCK cells was calculated as follow: CPR = (OD_{experiment} - OD_{virus control}) / (OD_{blank control} - OD_{virus control}) × 100%; virus inhibition rate of FG = (OD_{experiment} - OD_{virus control}) / OD_{virus control} × 100%.

Suppressive effect of FG on neuraminidase activity

The supernatant of influenza virus (bovine serum-free medium containing 1.2 mg/mL albumin, 5 µg/mL trypsin,) was added to MDCK cells. When cell changes were present, the supernatant was collected by centrifugation at 1,000 rpm for 10 min followed by inactivation with NP-40 (final concentration: 0.1%). The supernatant was filtered through a 0.22 µm filter serving as the neuraminidase solution, which was stored at 70°C (Peng et al., 2005; Li et al., 2009).

Inhibition for neuraminidase of FG

Neuraminidase (10 µL) was added into 96-well plate followed by addition of FG (20 µL) at different concentrations and subsequent incubation for 1 hours. After addition of 20 µmol/L MUNANA (40 µL) and 33 mmol/L MES (30 µL) (total volume: 100 µL),

Table I

Virus inhibition rate and cell protection rate following FG treatment *in vitro*

Group	Concentration (mg/mL)	Preventive action			Therapeutic action			Direct action		
		OD values	Cells protection rate / %	Virus inhibition rate %	OD values	Cells protection rate / %	Virus inhibition rate %	OD values	Cells protection rate / %	Virus inhibition rate %
FG	1.6	0.796 (0.029)	64.9	50.7	0.756 (0.023)	55.8	49.1	0.829 (0.023)	72.1	63.5
	3.1	0.808 (0.021)	67.4	59.4	0.763 (0.036)	57.4	50.5	0.843 (0.020)	75.3	66.3
	6.2	0.815 (0.025)	69.1	60.7	0.775 (0.021)	60.1	52.9	0.861 (0.031)	79.4	69.8
	12.5	0.826 (0.042)	71.5	62.9	0.785 (0.028)	62.3	54.8	0.871 (0.029)	81.6	71.8
Ribavirin	0.03	0.856 (0.035)	78.3	68.8	0.818 (0.034)	69.7	61.3	0.887 (0.016)	85.2	75.0
Blank	-	0.953 (0.050)	100.0	-	0.953 (0.050)	100.0	-	0.953 (0.049)	100.0	-
Virus	-	0.507 (0.043)	47.3	0	0.507 (0.043)	47.3	0	0.507 (0.043)	47.3	0

Data within the parenthesis is SD

incubation was performed at 37°C for 30 min. Then, 25% alcohol containing 0.1 mmol/L glycine (pH= 10.7; 200 µL) was added to stop reaction. Blank control was also included. In the positive control group (MES), peramivir was added. In the detection of neuraminidase activity, sample solution of same volume was added. In the control group, medium of same volume was added. Neuraminidase activity was determined with a fluorescence microplate reader with EX at 355 nm, EM at 460 nm, required value of 20% and gain value of 20. The inhibition rate (IR) was calculated (Mosmann, 1983). The half inhibitory concentration (IC₅₀) of FG on neuraminidase was calculated with the Bliss method (Nayak and Reichl, 2004).

Hemagglutination inhibition assay

PBS (pH 7.2) was added to a 96-well plate (25 µL/well), and 50 µL FG solution was added in the first tier which was dispensed by the serum (by RDE treatment, dilution of 1:10) and continuous multiproportion diluted until dumping 25 µL in the last one (No. 8). The first tier concentration was 50 mg/mL. Then 25 µL influenza virus (A/PR8/34) suspensions were added to each pore of 4 hemagglutination units, and it was covered at room temperature 1 hours after thoroughly miscene bene. Eventually, adding 0.75% guinea pig red blood cell suspensions 50 µL which were dispensed by PBS buffer in each pore, and laying the drug control group (an action of only drugs without added liquid of influenza virus), PBS buffer control group (only the influenza virus suspensions and PBS buffer without added drugs) and serum control group (only serum and

red cell suspensions of guinea pig), observing and recording after placed it in incubator for 60 min at 4°C.

Criteria for determination: (1)++++: Red blood cells arranged evenly on bottom; (2)+++ : Red blood cells arranged evenly on bottom but the edge of cell layer was thin; (3) ++: Red blood cells formed a circle on the bottom, which was surrounded by a small agglutination block; (4) +: Red blood cells formed a group on the bottom without smooth edge; (5)-: Red blood cells formed a group on the bottom with smooth and regular edge.

Results

When the concentration of FG was lower than 12.50 mg/mL, there was no significant difference in cell proliferation between the FG group and control group, indicating that FG at <12.5 mg/mL has no toxic effect on MDCK cells.

In the present study, the anti-viral effect of FG was determined by MTT assay and ribavirin treatment served as a positive control. Results showed FG could exert anti-viral effect to a certain extent in three groups but the anti-viral effect in Group C was the best and the anti-viral effect in Group A was superior to that in Group B. Anti-viral effect of FG is shown in Table I. 1) Group A: When compared with virus control group, the virus inhibition rate and cell protection rate following FG treatment were markedly increased (p<0.01). However, the virus inhibition rate and cell protection

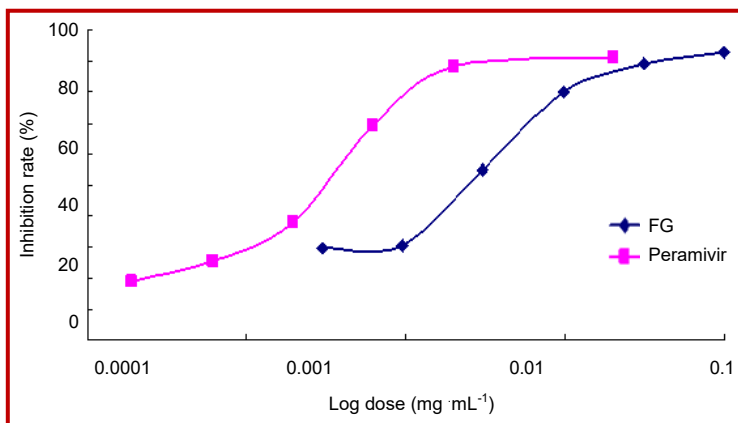


Figure 2: Dose-response relationship between FG and peramivir

Table II				
Inhibition of FG on hemagglutinin in influenza virus				
Concentration	Groups			
(mg/mL)	Experiment	Drugs	PBS buffer	Serum
50	+	-	++++	-
25	+	-	++++	-
12.5	++	-	++++	-
6.3	++	-	++++	-
3.1	+++	-	++++	-
1.6	++++	-	++++	-
0.8	++++	-	++++	-
0.4	++++	-	++++	-

rate following FG treatment were inferior to those in the positive control group ($p < 0.05$). 2) Group B: When compared with virus control group, the virus inhibition rate and cell protection rate following FG treatment were markedly increased ($p < 0.01$). However, the virus inhibition rate and cell protection rate following FG treatment were inferior to those in the positive control group ($p < 0.05$). 3) Group C: When compared with virus control group, the virus inhibition rate and cell protection rate following FG treatment were markedly increased ($p < 0.01$). However, the virus inhibition rate and cell protection rate following FG treatment were inferior to those in the positive control group ($p < 0.05$).

Suppressive effect on neuraminidase activity was observed following treatment with FG or peramivir. The dose-response curve between response rate and the Logarithmic dose was "S" shaped (Figure 2). The suppressive effect of FG was similar to that of peramivir. The IC_{50} of FG was 0.1 mmol/mL and that of peramivir was 0.003 nmol/mL.

FG had obvious inhibitory effect on influenza virus

hemagglutinin and the minimum inhibitory concentration was 3.1 mg/mL. Non-specific agglutination was absent following serum treatment. (Table II)

Discussion

Influenza is a communicable acute upper respiratory tract disease which is caused by influenza virus. It is highly contagious, can spread rapidly and often endemic. *R. isatidis* has been extensively used in China for over 2000 years, especially for anti-virus with definite clinical effectiveness. However, the selection of compounds for prevention or treatment of influenza virus infection is still limited and these compounds are susceptible to cause drug resistance and have side effects. Thus, to develop novel anti-viral drugs has been an important issue in clinical practice.

Co-culture of cells and virus and measurement of cell proliferation by MTT assay have been a main method to screen anti-viral drugs *in vivo*. To more objectively, sensitively and accurately evaluate the anti-viral effect of FG, MTT assay was also performed and FG was used for prevention and treatment of virus infection. Our findings suggested FG could exert suppressive effect on influenza A virus to a certain extent. The suppressive effect when cells were treated with FG and virus at the same time was the best and the suppressive effect in prevention of virus infection was also superior to that in treatment of virus infection. The *in vitro* cell protection rate and virus inhibition rate and virus inhibition rate of FG increased with the increase concentration of FG concentration, which were significantly different from those of cells with virus treatment alone ($p < 0.01$). Meanwhile, ELISA was performed to determine the suppressive effect of FG on neuraminidase activity in influenza A virus. Results showed the IC_{50} was 0.1 mmol/mL. Neuraminidase is a key enzyme affecting the replication, infection and

pathogenesis of influenza A virus. Thus, inhibition of neuraminidase activity can be used to control the symptoms and spreading of influenza A virus infection (Colman, 1994; Cao et al., 2002). Currently, substrate fluorescence detection (FL-MU-NANA method) is one of methods for the detection of neuraminidase activity *in vitro*, and has become an important method to screen and evaluate anti-viral activity of anti-influenza virus drugs including herbals (Peng and Li, 1999). In the present study, FL-MU-NANA method was employed to measure the biological activity of NA. Our results showed FG had anti-viral effect and neuraminidase may be one of targets of FG.

The influenza virus mainly consists of nucleocapsid and envelope. A glycoprotein in the envelope is a key protein to bind to receptors on cells of host and can form agglutination with red blood cells of animals. If the serum hemagglutinin antibody can specifically bind to the antigens on the virus, the interaction between hemagglutinin on virus and receptors on red blood cells is interrupted and red blood cell aggregation is then inhibited. In the present study, virus (hemagglutinin) was pre-treated with FG and then with red blood cells of guinea pig, and agglutination was observed.

Conclusion

Results showed FG at effective concentration could significantly inhibit the hemagglutinin of virus *in vitro*, and the minimum inhibitory concentration was 3.1 mg/mL.

Contributions

Sun Qin and Shui Pi Xian contributed equally in this study.

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Conflict of Interest

Authors declare no conflict of interest

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