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# Extraction and Separation of Polysaccharides from *Radix isatidis* and their Effects Towards Hemagglutinin Protein of Influenza Virus

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In this study, the anti-influenza virus effects of the polysaccharides of *Radix isatidis* and its possible target were explored. The hot water reflux extraction and stepwise ethanol precipitation were performed to extract and separate the polysaccharides from *R. isatidis*, the isolated constituents' activities against the influenza virus were subsequently evaluated through *in vitro* experiments. The ELISA assay was performed to verify the binding ability of 80 % alcohol-precipitated crude polysaccharides against the hemagglutinin (HA) of influenza A virus (A/PR/8/34, H1N1). The 70, 80 and 90 % alcohol-precipitated crude polysaccharides extracted from *R. isatidis* exhibited the anti-influenza virus activities,with the selection indexes (SI) as 2.69,7.52 and 5.75, respectively. Furthermore, the ELISA results showed that this constituent existed the specific binding capacity with the influenza virus HA with a dose-dependent manner. The 80 % alcohol-precipitated crude polysaccharides was one of the active components of this medicinal plant and the influenza virus HA protein might be its active target.

**Keywords: *Radix isatidis*, Polysaccharide, Isolation, Influenza virus hemagglutinin protein, Biological activity.**

## INTRODUCTION

The famous traditional Chinese medicine (TCM) *Radix isatidis*is the root of *Isatis indigotica* Fort, belonging to the Brassicaceae family. Various classes of compounds have been isolated from *R. isatidis*, including alkaloids (indirubin and indigo), saccharides (glycoproteins and polysaccharides) and lignans (isolariciresinol), which have been proved of such bioactivities as anti-bacteria, anti-virus, anti-endotoxin, anti-tumor and immuno modulatory effects by the modern pharmacological experiments[1-6]. Particularly, the role of anti-influenza virus has been a focus of study for *R. isatidis* for a long period. Currently, the anti-influenza virus drugs in clinical application mainly include M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir), but the resistance of influenza A virus strains towards M2 ion channel inhibitors has become very common[7]. Almost 100 % seasonal influenza A viruses (H1N1 and H3N2) worldwide in 2007-2008[8] and the H1N1 influenza virus in 2009 were resistant to adamantine[9,10]. In contrast to the western anti-viral drugs, the ones derived from traditional Chinese medicine often act on multiple viral targets, which makes them less prone to induce the drug resistance and more advantageous than the western drug compounds. However, the existing literatures and patent reports have not clarified the material basis of *R. isatidis* yet and in particular, the anti-viral mechanism of polysaccharides from *R. isatidis* required further exploration.

In our previous study[11], a crude extract (G2) from *Isatis indigotica* root was found to be able to inhibit different subtypes of human or avian influenza viruses. And the polysaccharides inside seemed to be the major constituents. Therefore, in this study, the polysaccharides were extracted and isolated from *R. isatidis* through the traditional water reflux extraction and ethanol precipitation methods[12]. Furthermore, the *in vitro* anti-influenza virus activities of the isolated polysaccharides was tracked and the possible mechanism was preliminarily explored, aiming to provid eadditional evidence to clarify the material basis of *R. isatidis* and explore the mechanism of its anti-viral effects.

## EXPERIMENTAL

Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS, Australian origin) and phosphate-buffered saline (PBS) were purchased from Gibco (USA). HPD 600 macroporous resin was purchased from Hebei Bonherb technology Co., Ltd. (China); KBTES lyophilizer was from VIRTIS (USA); Agilent 1200 series HPLC was from Agilent (USA); Alltech 3300 ELSD detector was from Grace (USA) and TSK G3000PW gel column was obtained from TOSOH (Japan).

**Drug, plant material, viruse and cells:** The positive control drug ribavirin was purchased from Guangdong Zhaoqing Star Lake Bioscience Co., Ltd. (lot number: L20120325). *R. isatidis* was provided by Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co., which was originally collected from Daqing *R. isatidis* GAP base and identified as the root of *Isatis indigotica* Fort by Huaguo Ye, researcher at the South China Botanical Garden of the Chinese Academy of Sciences.

The PR8 strain (A/PR/8/34, H1N1) of influenza A H1N1 virus was purchased from the American Type Culture Collection (ATCC). The viruses were propagated in 9-11 days old chicken embryos, the allantoic fluid was harvested for the hemagglutination titer determination. The Reed-Muench method[13] was applied to measure the TCID50 (50 % tissue culture infective dose), which was then used as the virus titer for the anti-cytopathic effect assay.

The canine kidney cells (Madin Darby Canine Kidney, MDCK) were from the Type Culture Collection cell bank of Chinese Academy of Sciences. All the cells were cultured in 10 % FBS-containing DMEM .

**Preparation and analysis of polysaccharides:** Preparation of crude polysaccharides: 10 kg of *R. isatidis* dry herbs were weighed and ground, then the fine powder was subjected to degreasing with 1 h reflux with 4-fold petroleum ether (w/v) as the solvent. After it was filtered and dried, the herb grounds were added to 10-fold hot water (w/v) for 2 h reflux extraction (twice). The aqueous extractions were then combined, filtered and concentrated under reduced pressure until the final volume was 1/7-1/8 of the original. The concentrated herb solution was sequentially precipitated with 50, 60, 70, 80 and 90 % ethanol to get different precipitations, generating 5 fractions as E1, E2, E3, E4 and E5. The phenol-sulfuric acid method was used to detect the polysaccharide contents of the above fractions and the gel permeation chromatography and evaporative light scattering detection (GPC-ELSD) method was used to determine the molecular weight distributions.

**Drug toxicity test (MTT method):** The MTT assay was performed to determine the toxicity of the isolated compounds towards MDCK cells. The pharmacological experiments were conducted at the maximum non-toxic concentrations[14] and the Reed-Muench method[13] was used to calculate the concentration of 50 % drug toxicity ($TC_{50}$).

**Determination of *in vitro* anti-influenza virus activity:** The *in vitro* cell model was used for the investigation of the anti-viral activity of various fractions. The MDCK cells were seeded into 96-well plate and incubated at 37 °C and 5 % $CO_2$ for 24 h. After washed with 1 × PBS, the influenza virus solution of 100 $TCID_{50}$ (concentration that would infect 50 % cells) was added. The supernatant was discarded after 2 h incubation at 37 °C and 5 % $CO_2$ and the various fractions were diluted 2-fold by the serum-free TPCK trypsin-containing MEM and added into the plate with the concentration as 2 μg/mL. After incubated for another 48 h at 34 °C and 5 % $CO_2$, the cytopathic effects of the groups were observed under the microscope and recorded. The Reed-Muench method[13] was used to calculate the 50 % inhibitory concentration[14] ($IC_{50}$) and the selection index (SI) was defined as $TC_{50}/IC_{50}$[15].

Interactions between *R. isatidis* crude polysaccharides and influenza virus HA protein by ELISA method

The influenza virus HA protein was used as a coating antigen after 1250-fold dilution,100 μL was added into each well of a 96-well V type plate. After the overnight incubation at 4 °C and subsequent washing, 400 μL/well 2 % BSA-containing PBS with Tween-20 (PBST) was added for 2 h incubation. After washing,100 μL/well of various drugs at their appropriate concentrations were then added for 2 h incubation at 37 °C. Then 100 μL diluted primary antibody was added for 1 h incubation at 37 °C; after washing, 100 μL secondary antibody dilutedas 1:4000 was added for 1 h incubation at 37 °C. After that, the wells were washed and the substrate solutions of A and B were added. After 10-min chromogenic reaction at room temperature, the reaction was terminated and the absorption values at 450 nm (A450) was detected with a microplate reader.

A mixed solution of the subject sample, together with a specific amount of enzyme-labeled antibody solution, was added into the tube for the testing, allowing a reaction with the solid-phase antigen. If the subject samples did not contain any antibody, the enzyme-labeled antibody then could successfully interact with the solid-phase antigen. If the subject samples contained the antibodies, the antibodies would have the same chance of interacting with the solid-phase antigen as the enzyme-labeled antibody, which competitively reduced the opportunity of the enzyme-labeled antibody's interacting with the solid-phase antigen and reduced the amount of enzyme-labeled antibody's bound to the solid-phase antigen. The enzyme-labeled antibody was only added to the control tube; therefore, after the incubation, the interaction between the enzyme-labeled antibody and solid-phase antigen could reach a maximal level. After washing, the chromogenic substrate was added and the control tube would exhibit the darkest colour due to having the highest amount of bound enzyme-labeled antibodies. The colour difference between the control tube and the sample tube represented the amount of antibodies in the sample. The lighter the colour of the test tube, the higher the antibody level in the sample[16].

## RESULTS AND DISCUSSION

**Preparation of polysaccharides:** In this study, we sequentially adjusted the alcohol concentration in the extract solution to 50, 60, 70, 80 and 90 %, and obtained five types of crude polysaccharide with different molecular weight distributions: 50 % alcohol-precipitated crude polysaccharide constituent (E1, 1670 g extractum, yield rate as 16.7 %), 60 % alcohol-precipitated crude polysaccharide constituent (E2, 80 g extractum, yield rate as 0.8 %), 70 % alcohol-precipitated crude polysaccharide constituent (E3, 160 g extractum, yield rate as

1.6 %), 80 % alcohol-precipitated crude polysaccharide constituent (E4, 410 g extractum, yield rate as 4.1 %) and 90 % alcohol-precipitated crude polysaccharide constituent (E5, 200 g extractum, yield rate as 2 %). The results of phenol-sulfuric acid chromogenesis were significant, indicating that the five fractions all contained the sugar compounds. The gel chromatography results of E3 and E4 fractions obtained on GPC-UV-ELSD detection were shown in Figs. 1 and 2.

Dextran standards with different molecular weights were used to make a solution at approximately 10 mg/mL for the gel chromatography. The standard curve was plotted with the retention time (t/min) as the horizontal coordinate and the log ($M_w$) as the vertical coordinate. The regression equation was as the following: log ($M_w$) = 9.87-0.59t and the correlation coefficient was r = 0.993. According to the retention times of various chromatographic peaks of the above fractions, the molecular weight distributions of the above fractions were calculated. The traits, yields and molecular weight distributions of the above fractions were shown in Table-1.

As shown in Table-1, with the decreased molecular weights, the water-solubilities of the fractions increased. With respect to the molecular weight distributions, the gradient alcohol precipitation achieved the crude separation and as the ethanol concentrations increased, the molecular weight distribution stended to favor small molecules. As shown in Figs. 1 and 2, the chromatographic peaks at about 10 min (molecular

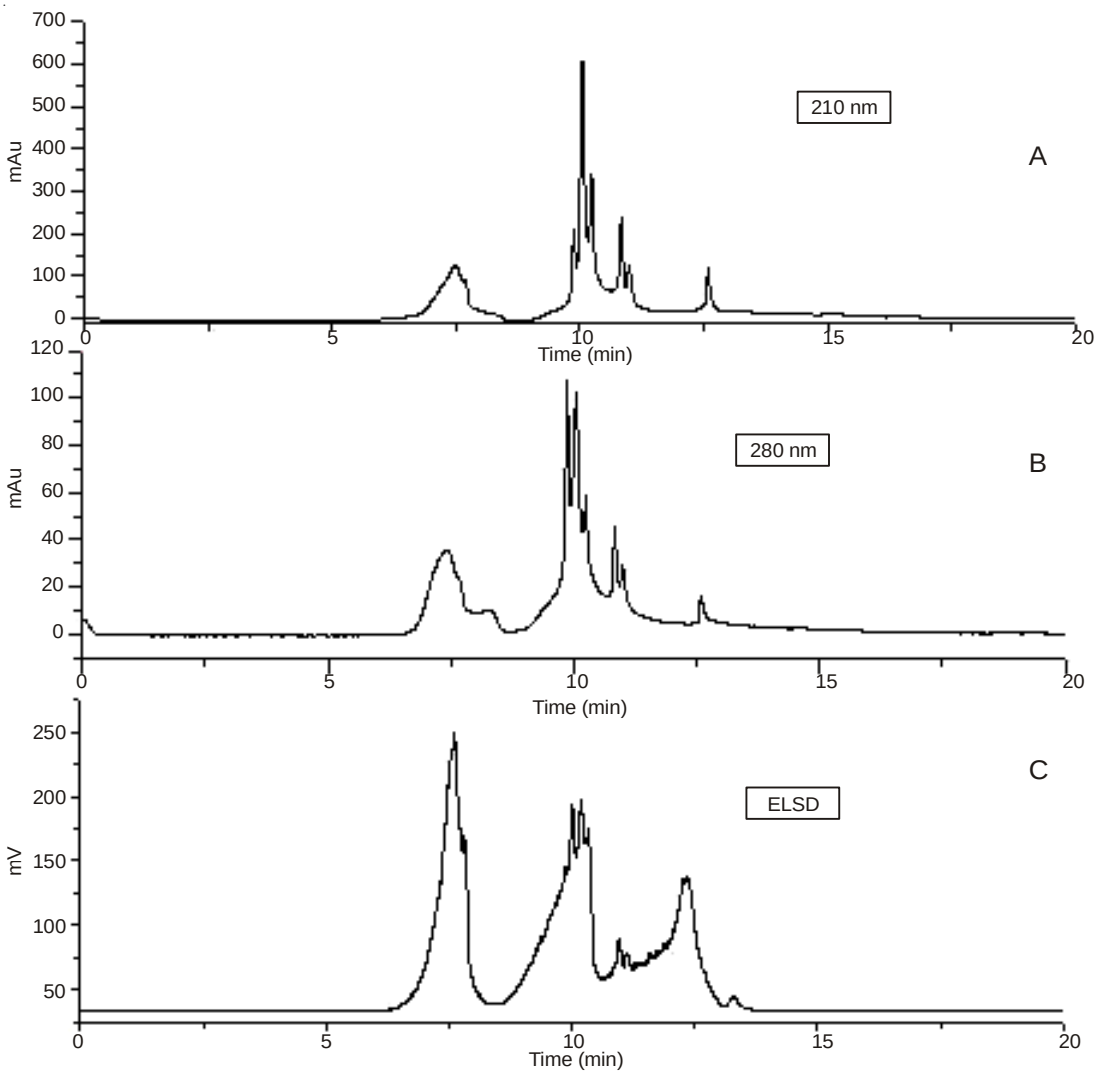


Fig. 1. Gel chromatography of E3 fraction; A: UV detection at 210 nm; B: UV detection at 280 nm; C: ELSD detection

TABLE-1
ANALYSIS OF VARIOUS FRACTIONS OBTAINED FROM THE GRADIENT ALCOHOL PRECIPITATION

| Sample | Traits | Solubility | Yield (%) | Distribution of main molecular weight | Content of sugar |
|---|---|---|---|---|---|
| E1 | Gray-white powder | Minimally dissolved in water | 16.7 | >150000 | Insoluble |
| E2 | Milky white powder | Partly dissolved in water | 0.83 | >150000, 17000, 2000 | 17.3 % |
| E3 | Brown powder | Dissolved in water but remained slightly turbid | 1.63 | 24000, 5000 | 19.0 % |
| E4 | Brown-yellow powder | Easily dissolved in water | 4.08 | 22000 | 18.3 % |
| E5 | Light yellow powder | Easily dissolved in water | 1.98 | 29000, 1300 | 28.0 % |
| Supernatant extractum | Dark brown paste | Easily dissolved in water | 11.83 | Less than 1300 | Minute content |

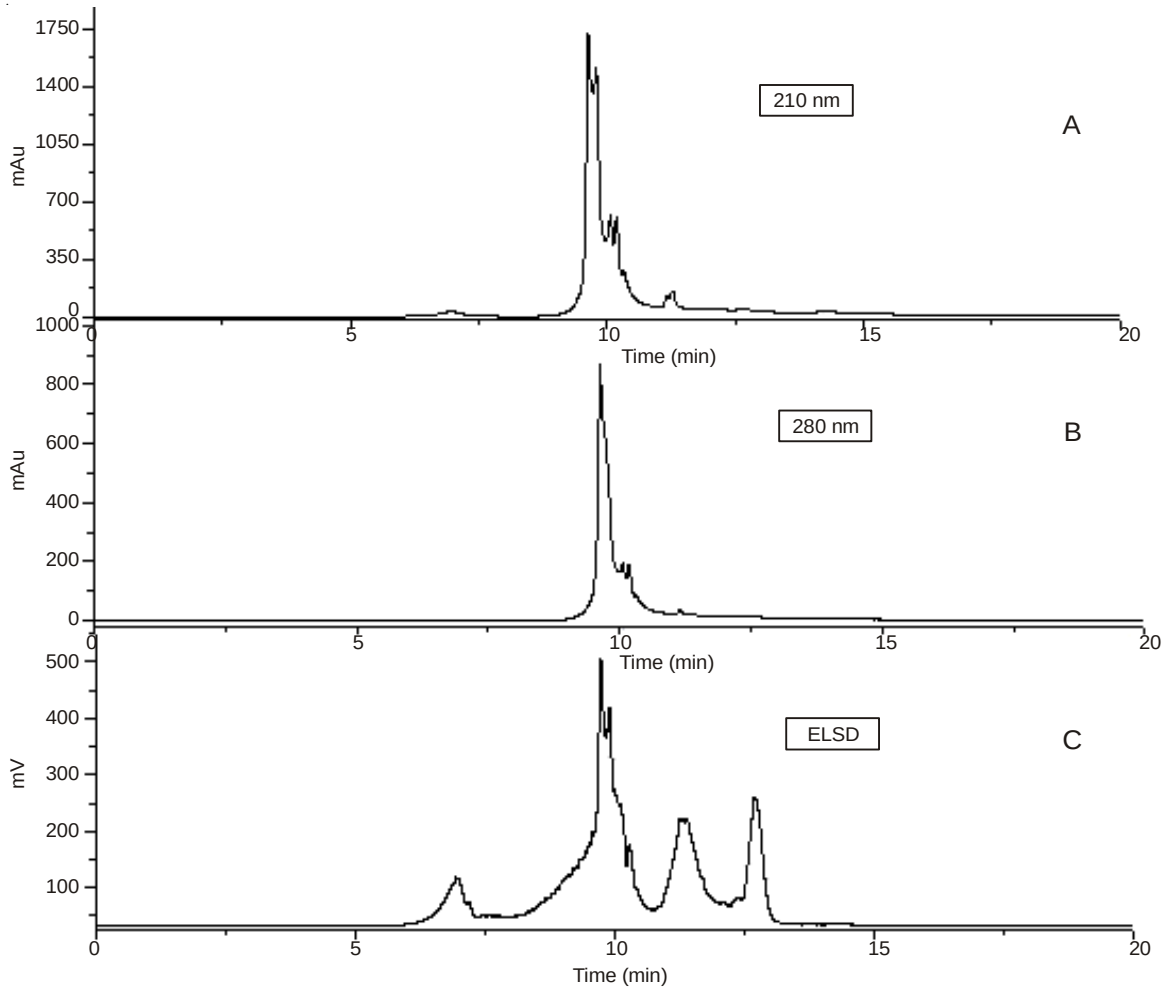


Fig. 2. Gel chromatography of E4 fraction; A: UV detection at 210 nm; B: UV detection at 280 nm; C: ELSD detection

weight was at approximately 20,000 Da) showed the relatively strong UV absorption at both 210 and 280 nm and after eliminating the proteins, the gel chromatograms of these crude polysaccharides showed no changes, suggesting that the compound with a molecular weight at approximately 20,000 might be a glycopeptide.

**Cytotoxicity:** To test the anti-viral efficacy, we tested the toxicity of the polysaccharide drug samples on the virus host cells. The $TC_{50}$ of *R. isatidis* polysaccharides was in the range of 0.25-37.6 mg/mL as measured by MTT assay (Table-2).

***in vitro* Anti-influenza virus activity:** The cytopathic effect-inhibition assay was performed to investigate the anti-viral activities of the obtained fractions. The results showed that the E3, E4 and E5 fractions all exhibited anti-viral activities against the PR8 strain of influenza A (H1N1) viruses with $IC_{50}$s as 3.83,5 and 3.34 mg/mL, respectively. The E4 showed the strongest effect (SI 7.52) (Table-2).

**Validation of inhibitory effects of E4 aganist influenza virus HA protein by ELISA:** The purpose of this experiment was to determine whether the influenza virus PR8-HA protein and E4 could bind each other. The primary antibody concentration was fixed at 1 μg/mL and E4 was diluted from 5 mg/mL in two-fold serial dilutions to 0.078125 mg/mL, the values were expressed as mean ± SD for three repeated experiments. The results showed that the OD values increased accordingly with the diluted concentrations, with the correlation coefficient of $-0.983 < P < 0.01$, indicating that E4 might have a specific capacity to bind PR8-HA and the greater the concentrations, the stronger the binding capacities, indicating that HA might be the target that E4 bound within the influenza viruses (Fig. 3).

TABLE-2
ANTI-VIRAL EFFECTS SCREENING OF VARIOUS FRACTIONS

| Constituent | $TC_{50}$ (mg/mL) | $IC_{50}$ (mg/mL) | SI ($TC_{50}/IC_{50}$) |
|---|---|---|---|
| Supernatant of alcohol precipitation | – | > 1 | < 1 |
| E1 | < 5 | > 5 | < 1 |
| E2 | < 5 | > 5 | < 1 |
| E3 | 10.3 | 3.83 | 2.69 |
| E4 | 37.6 | 5 | 7.52 |
| E5 | 19.2 | 3.34 | 5.75 |
| Petroleum ether-degreased constituent | < 0.25 | > 2 | < 1 |

"–" Undetected

In recent years, studies of the anti-viral effects of *R. isatidis* have mainly focused on exploring its material basis. Among the constituents with anti-viral activities, many were concentrated in the highly polar fraction, especially the *R. isatidis* polysaccharides become the highly concentrated in this fraction. According to Yamada *et al.*[17], *R. isatidis* polysaccharides and glycoproteins had anti-viral activities and could induce the generation of IgG antibodies against the influenza

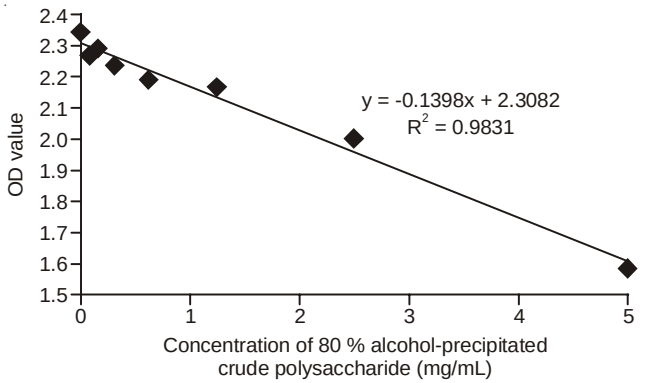


Fig. 3. ELISA results of E4

viruses. *R. isatidis* polysaccharides could also boost the immune function of mice[18] and enhance the ability of mice to resist influenza virus infection. In addition, *R. isatidis* polysaccharides, together with their protein components, might bind the cell surface sialic acid and there by compete with the influenza viruses in the aspects of adsorption, thus protecting the cells[19, 20]. Therefore, in this study, we focused the material basis on the *R. isatidis* polysaccharide and further explored its anti-influenza virus activities and the possible mechanism. Our study could be beneficial to the improvements of production process, quality control and new drug development.

In this study, the hot water reflux was used to extract *R. isatidis* polysaccharides. Prior to the aqueous extraction, the herbs were treated with petroleum ether to prevent the interference by small weakly polar molecules (such as the volatile oils) in *R. isatidis*. Ethanol precipitation was a simple method to obtain polysaccharides from an extract solution. Different concentrations of ethanol could be used to precipitate polysaccharides with different characteristics or molecular weights. According to the gel chromatograms, the compounds of E3 and E4 showed relatively strong UV absorption at both 210 and 280 nm and the molecular weights were approximately 20,000, indicating the existence of glycopeptide structures in these fractions. Based on the pharmacological results of various fractions, the E4 exhibited the best anti-influenza virus activity (SI was up to 7.52). We thus, further explored the anti-viral mechanism of this fraction.

Influenza virus is an enveloped single-stranded negative RNA virus. The glycoprotein HA is the surface antigen of influenza viruses that could be recognized and bound by the receptors on the surface of host cells. After being hydrolyzed into HA1 and HA2 by cellular trypsin, glycoprotein HA could fuse with the cell membrane, releasing the viral genome into the cytoplasm and initiating replication[21]. Thus, HA is a decisive factor for the process that the influenza virus uses to infect the host cells. In the early stages of viral replication, HA plays a key role inviral adsorption and penetration and thus the inhibition of its adsorption or fusion activity could effectively inhibit the replication of influenza virus. Therefore, both HA1 and HA2 could become novel targets for new anti-influenza virus drugs[22].

Based on the anti-influenza virus activities of *R. isatidis* polysaccharides, the present study used the competitive ELISA method to verify the binding affinity of *R. isatidis* polysaccharides towards the influenza virus HA protein. The results showed that E4 exhibited a specific binding capacity towards the HA antigen at a concentration of 5 mg/mL and the binding capacity was positively dose-dependent. Therefore, the E4 could compete with the influenza viruses for the host cell HA receptors, thus preventing the fusion of the viruses with the cells. In our previous studies, we found that a polysaccharide isolated from the high-polarity fraction of *R. isatidis* had the effects of inhibiting influenza virus adsorption and invasion and its target within the influenza virus was HA[11]. Although *R. isatidis* polysaccharides had satisfactory anti-viral activities, the majority of existing studies had used the crude polysaccharides as the test materials and the purities of crude polysaccharides were insufficient to achieve the purity of drug compounds; moreover, the chemical structure of the secrude polysaccharides had not been elaborated. In addition, mice that were intraperitoneally injected the *R. isatidis* polysaccharides showed the enhanced *in vivo* IgG levels and IFN-γ levels inside the spleen cells[18]. However, *in vivo* metabolism of *R. isatidis* polysaccharides and the effects of their metabolites still remained to be further explored.

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