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Comparison of Bush Sophora Root polysaccharide and its sulfate's anti-duck hepatitis A virus activity and mechanism

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ABSTRACT

In order to research the sulfating modification in enhancing the anti-duck hepatitis A virus (DHAV) activity of Bush Sophora Root polysaccharide (BSRPS), sulfated Bush Sophora Root polysaccharide (sBSRPS) was prepared by chlorosulfonic acid–pyridine method. KBr pellets method was applied to analyze their different structures. Anti-DHAV activity was studied by duck embryonic hepatocytes culture in vitro and artificial inoculation method in vivo. Direct immunofluorescence method and Real-time PCR were applied to study the antiviral mechanism of adsorption, replication and release in vitro and the dynamic change of virus content of blood in vivo. The results showed at the most effective content, sBSRPS (7.813 μg/mL) could inhibit both replication and release of DHAV in vitro, BSRPS (500 μg/mL) only inhibit replication. The relative expression of DHAV gene at the 8th h and the mortality rate of sBSRPS group were significantly reduced. These results indicated sBSRPS performed more effectively in anti-DHAV activity than BSRPS.

1. Introduction

Duck virus hepatitis (DVH) is a serious virus disease of young ducklings with rapid transmission, high morbidity and high mortality which caused by duck hepatitis virus (DHV). This virus was first isolated on Long Island in 1949 (Levine & Fabricant, 1950) and is distributed worldwide at present. DHV is mainly divided into three serotypes including duck hepatitis virus type 1 and its mutant, duck astrovirus and duck hepatitis virus type 3. Duck hepatitis virus type 1 was renamed duck hepatitis A virus (DHAV) according to sequence analysis (Pan et al., 2012). Among these serotypes, DHAV is the most widely distributed and most virulent one. DHAV mainly infects young ducklings aged under three weeks and the mortality rate is higher than 80% (Tseng, Knowles, & Tsai, 2007). Currently, DVH caused by DHAV is one of the most serious diseases which damage the interest of duck industry.

There are no effective antiviral drugs against DHAV in clinical so far. Immunizing laying ducks or young ducklings through injecting of attenuated DHAV vaccine is the main way to control this disease. It would cause irreparable damage once the clinical cases emerged. Gerber et al. first discovered the antiviral activity of seaweed polysaccharides in 1958 (Gerber, Dugene, Adams, & Sherman, 1958). Hereafter, the antiviral activities of varieties of polysaccharides and sulfated polysaccharides have been reported successively (Dolores & Carmen, 1991; Lee et al., 2002; Kim et al., 2012; Song et al., 2013). A variety of polysaccharides of traditional Chinese medicine and its sulfates showed significantly resistance to the infections of Newcastle disease virus, infectious bursal disease virus and other viruses in our previous experiments (Nguyen et al., 2012; Wang, Hu, et al., 2010; Zhao et al., 2011).

Bush Sophora Root is a well-known traditional herbal medicine in China used for centuries to clear away the heat-evil, detoxify, reduce swelling and alleviate pain (Liu & Xu, 2011). Contemporary pharmacologic research showed that Bush Sophora Root polysaccharide (BSRPS) could resistant to oxidation effectively (Chen, Hu, & Zheng, 2007; Shuai et al., 2010). The sulfated modified product of BSRPS, sulfated Bush Sophora Root polysaccharide (sBSRPS) showed fine inhibition of hepatitis virus in vitro (Zhang, Jiang, Zhang, Luo, & Li, 2011). In this paper, we reported on the sulfated modification of BSRPS which modified by the chlorosulfonic acid–pyridine method and the characterization of BSRPS's and sBSRPS's antiviral efficacy against DHAV in vivo and vitro. In order to screen the preliminary ingredient of Chinese herb medicinal ingredient (CHMI) prescriptions of anti-DVH, BSRPS was modified

Abbreviations: DVH, duck virus hepatitis; DHV, duck hepatitis virus; DHAV, duck hepatitis A virus; BSRPS, Bush Sophora Root polysaccharide; sBSRPS, sulfated Bush Sophora Root polysaccharide; CHMI, Chinese herb medicinal ingredient; DMEM, Dulbecco's modified Eagle's medium; MM, maintenance medium; D-Hank's, Dulbecco's Hanks balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Pyr, pyridine; CSA, chlorsulfonic acid; DEHs, duck embryonic hepatocytes; CMF, calcium and magnesium-free; PBS, phosphate-buffered saline; CC, cell control; VC, virus control; BC, blank control; Real-time PCR, real-time polymerase chain reaction; PBST, phosphate buffered saline Tween-20.

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by the chlorosulfonic acid–pyridine method. The characterization of BSRPS's and sBSRPS's antiviral efficacy against DHAV in vivo and in vitro and their mechanisms were comparatively investigated in present experiment.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with penicillin 100 IU/mL, streptomycin 100 IU/mL, 10% fetal bovine serum, insulin 1 μg/mL and glutamine 0.75 mg/mL was used as nutritive medium. For maintenance medium (MM), the fetal bovine serum concentration was reduced to 1%. Dulbecco's Hanks balanced salt solution (D-Hank's) was used for washing the embryo tissue fragments and cells. D-Hank's, DMEM and MM, pH was adjusted to 7.4 using 5.6% $NaHCO_3$. Trypsin (Amresco) was dissolved into 0.20% with D-Hank's. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco) was dissolved with calcium and magnesium-free phosphate-buffered saline into 5 mg/mL. These reagents were filtered through 0.22 μm syringe filters. DMEM and MM were stored at 4 °C and MTT solution was stored at 4 °C in dark bottles. Other chemicals used in the experiment were analytical grade.

Pyridine (Pyr, Lot no. T20110825) was bought from Sinopharm Group Chemical Company. Chlorsulfonic acid (CSA, Lot no. 080421) was the product of Shanghai Ling Feng Chemical Company. RNAiso Plus Reagent (Lot no. 9108), PrimeScript™ RT Master Mix Kit (Lot no. RR036A) and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit (Lot no. RR036A) were bought from Takara. Fluorescein isothiocyanate-labeled rabbit anti-DHAV (Lot no. orb8860) was bought from biorbyt.

2.2. Preparation of BSRPS and its modified product

2.2.1. Extraction of BSRPS

Water-extraction and alcohol–precipitation method was applied to BSRPS's extraction (Zhang, Hu, et al., 2012). 1000 g of Bush Sophora Root (Bought from Jingui Chinese Herbal Medicine Company, Hubei province, China, Lot no. 121001) was soaked with 10-fold water for 3 h and then decocted three times for 1 h per time. Supernatant was extraction after centrifugation of the condensation of extraction. 95% ethanol was added into the abovementioned supernatant to make ethanol concentration reach to 75% (v/v). After 24 h, the precipitation was dried at 60 °C and 46.49 g BSRPS was obtained. It was purified through eliminating protein by Sevage method and column chromatography of Sephadex G-200 (2 cm × 100 cm) and then eluted with distilled water (Qin et al., 2013). Its final polysaccharide content was 83.77% determined by phenol–sulfuric acid method (Hsieh, Hsu, & Yang, 2005).

2.2.2. Preparation of sBSRPS

Chlorosulfonic acid–pyridine method was applied to prepare sBSRPS (Guo et al., 2009). The conditions for modification of sulfation modification were 2 h of the reaction time, 95 °C of the reaction temperature and 1:5 of the ratio of CAS to Pyr. The polysaccharide content of sBSRPS was determined by phenol–sulfuric acid method while sulfur content was determined by barium chloride–gelatin method (Dodgson & Price, 1962). The content of sBSRPS was 89.18% which calculated with the sum of its polysaccharide content and sulfur content.

2.2.3. Infrared spectroscopy analysis

The FT-IR spectrum of BSRPS and sBSRPS in a wavenumber range of 4000–400 cm^{-1} was recorded by KBr pellets method (Qin et al., 2013) with a Nicolet 200 Magna-IR spectrometer (Nicolet Instrument Corp.). The major peaks (intensity and wavenumber) were found using OMNIC software (Nicolet Instruments Corp.).

2.3. Duck embryonic hepatocytes (DEHs) and DHAV

Livers were removed from 14 to 16 days duck embryo aseptically followed by removing gallbladder (Sauerbrei et al., 2005). Afterwards, livers were washed three times with D-Hank's. The tissue was minced and subsequently washed three times with D-Hank's. Liver tissue was digested with a solution of 0.20% trypsin for 9 min. Washed the tissue three times with D-Hank's after removing the trypsin and then cultured in DMEM. Made sure the seeding density of cells was 0.8×10^6–1.2×10^6 cells/mL and cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 and the growth medium replaced after 24 h (Schacke, Glück, Wutzler, & Sauerbrei, 2009). When duck embryonic hepatocytes grew into monolayer, DMEM was removed. The DEHs monolayer was washed with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4) once, and taken for standby after CMF-PBS removed.

DHAV (LQ_2D_7 strain) used for challenge experiment and antiviral assays was supplied by the Shandong Institute of Poultry in China and proliferated by inoculating DEHs. $TCID_{50}$ of the virus liquid was 1×10^{-3} by Reed–Mueech assay (Huang et al., 2008). It was diluted into 5×10^{-2} (50 $TCID_{50}$) with MM and used for antiviral assays.

2.4. Antiviral activity of BSRPS and sBSRPS

2.4.1. The antiviral activity in vitro

BSRPS was diluted according to the net content with MM 2-fold serially from 2000 μg/mL to 250 μg/mL, sBSRPS, from 7.813 μg/mL to 0.977 μg/mL, total 4 concentrations, respectively (the maximum safe concentration of BSRPS was 2000 μg/mL tested by preliminary experiment while sBSRPS's was 7.813 μg/mL). 70 μL DHAV was added to each well of 96-well plate containing a DEHs monolayer except for what treated as cell control (CC) group. And the plate was incubated at 37 °C in a humid atmosphere of 5% CO_2 for 2 h. The virus solution was removed, and the monolayer was washed thrice with D-Hank's. Hereafter, added 70 μL dilutions of test polysaccharides to test wells, five repetitions for per concentration. And the plate was continued to be incubated at 37 °C. At the same time, established CC group and virus control (VC) group. Measured the DEHs livingness by MTT colorimetric assay (Zhang, Sun, et al., 2012) when the VC group appeared obviously cytopathic effect (approximately after 72 h). The virus inhibitory rate was calculated based on the formula: virus inhibitory rate $= (\bar{A}_{\text{drug+virus}} - \bar{A}_{\text{virus control}})/(\bar{A}_{\text{cell control}} - \bar{A}_{\text{virus control}}) \times 100\%$ (Takeuchi, Baba, & Shigeta, 1991). The A_{570} values and virus inhibitory rate were considered as the indicators of antiviral activity.

2.4.2. The antiviral activity in vivo

2.4.2.1. Animals grouping and treatment. 180 three-day-old cherry valley ducks (Purchased from Tangquan Poultry Farm, Jiangsu province, China) were randomly divided into four groups. Group 1 to group 4 were respectively treated as BSRPS treated group, sBSRPS treated group, VC group and blank control (BC) group (isolation reared). Young ducklings of group 1–3 were intramuscular injected DHAV virus 0.2 mL per feather. 1 h later, the ducklings in BSRPS treated group was treated by aqueous BSRPS solution at the dosage of 5 mg per feather according to the net content while the sBSRPS treated group was treated by aqueous sBSRPS solution at the dosage of 2.5 mg per feather according to the net content, once a day for three days, respectively. The dosage of drugs was

determined by the effective concentration against DHAV in vitro and was effective in vivo according to preliminary test. To monitor the blood virus contents in emergency (4th h and 8th h) and stable (72nd h) phases of the disease, blood samples randomly taken from 5 feathers per group at the 4th, 8th and 72nd h after challenged virus were collected, treated as heparin anticoagulation and used for testing its virus nucleic acid contents.

The pathogenic and dead statuses of ducks were clinically examined daily for 5 successive days after the challenge until no death was found. Once duck death was found, examined the pathology changes carefully and eliminated the number of the ducks without pathology changes.

2.4.2.2. Quantitative analysis of DHAV in the blood. Real-time polymerase chain reaction (Real-time PCR) was applied to quantitative analysis the dynamic change of DHAV in vivo.

2.4.2.3. The clinical curative effects of BSRPS and sBSRPS. Until no death was found in all the groups, the mortality in every group was calculated according to the formula: Mortality (%) = the number of dead ducks/the number of sample × 100%. Number of ducks which had been taken blood samples needed to eliminate (15 feathers per group). Time of death ending was recorded.

2.5. Mechanisms underlying the effect of antiviral activity of BSRPS and sBSRPS in vitro

2.5.1. Virus adsorption assay

Two sample-adding modes were applied to assay the adsorption by direct immunofluorescence method (Carraro et al., 2007; Iwamoto, Burrows, Born, Piepkorn, & Bothwell, 2000).

Post-adding drug: 70 μL DHAV was added to each well of 96-well plate containing a DEHs monolayer except for what treated as CC group, and the plate was incubated at 37 °C in a humid atmosphere of 5% CO_2 for 1.5 h. The virus solution was removed, and the monolayer was washed thrice with D-Hank's. Hereafter, 70 μL dilution of test polysaccharide at the most effective antiviral concentration was added into test wells except for that CC group and VC group, three wells repeatedly. And the plate continued to be incubated at 37 °C for 1.5 h.

Pre-adding drug: 70 μL dilution of test polysaccharide at the most effective antiviral concentration was added into test wells of 96-well plate containing a DEHs monolayer except for what treated as CC group and VC group, three wells repeatedly, and the plate was incubated at 37 °C in a humid atmosphere of 5% CO_2 for 1.5 h. The compounds were removed, and the monolayer was washed thrice with D-Hank's. Hereafter, 70 μL DHAV was added into each well except for what treated as CC group. And the plate was continued to incubate at 37 °C for 1.5 h.

Afterwards, plates were washed five times with phosphate buffered saline Tween-20 (PBST) and fixed with paraformaldehyde at 4 °C for 20 min followed by washing plates five times with PBST. Plates were incubated with fluorescein isothiocyanate-labeled rabbit anti-DHAV at 37 °C for 1 h. Then plates were finally washed ten times with PBST and directly observed the brightness of fluorescence by a fluorescence microscope (HBO 100, ZEISS, Japan).

2.5.2. Virus replication assay

400 μL DHAV was added to each well of 24-well plate containing a DEHs monolayer except for what treated as CC group, and the plate was incubated at 37 °C in a humid atmosphere of 5% CO_2 for 2 h. The virus solution was removed, and the monolayer was washed thrice with D-Hank's. Hereafter, 400 μL dilution of test polysaccharide at the most effective antiviral concentration was added into test wells, three wells repeatedly. At the same time, established CC group and VC group. And the plate continued to be incubated at 37 °C for 24 h. When total RNA was extracted, Real-time PCR was employed to quantify the amount of virus replication according to the following method.

2.5.2.1. Primer design. The primers based on the complete genome of DHAV (ZJ strain; GenBank: EU841005) were designed using Primer Premier Software (version 5.0). The forward and reverse primers were 5′-GCCACCCTTCCTGAGTTTGT-3′ (positions: 3336–3355) and 5′-TACCATTCCACTTCTCCTGCTT-3′ (positions: 3489–3510).

Beta-actin of ducks was chosen as control genes. The primers based on the complete coding sequence of anas platyrhynchos beta-actin mRNA (GenBank: EF667345) were designed using Primer Premier Software (version 5.0). The forward and reverse primers were 5′-CTTTCTTGGGTATGGAGTCCTG-3′ (positions: 826–847) and 5′-TGATTTTCATCGTGCTGGGT-3′ (positions: 995–1014).

2.5.2.2. RNA extraction and reverse transcription. Total RNA was extracted by using RNAiso Plus Reagent according to the manufacturer's instructions. Made sure its OD_{260}/OD_{280} was between 1.8 and 2.1. Reverse transcription assay by using PrimeScript™ RT Master Mix Kit was reacted in the PCR instrument (2720 Thermal Cycler PCR instrument, Applied Biosystems, American). Reverse transcription was performed at 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 7 min.

2.5.2.3. PCR. Real-time PCR by using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit was reacted in the PCR instrument (StepOnePlus™ Real Time PCR instrument, Applied Biosystems, American). Real-time PCR was performed at 95 °C for 30 s, 95 °C for 5 s (40 cycles), and 60 °C for 30 s.

2.5.3. Virus release assay

400 μL DHAV was added to each well of 24-well plate containing a DEHs monolayer except for what treated as CC group, and the plate was incubated at 37 °C in a humid atmosphere of 5% CO_2 for 2 h. The virus solution was removed, and the monolayer was washed thrice with D-Hank's. Hereafter, 400 μL dilution of test polysaccharide at the most effective antiviral concentration was added into test wells, three wells repeatedly. At the same time, established CC group and VC group. And the plate continued to be incubated at 37 °C for 48 h. The supernatant of cells was centrifuged and the sediment (cellular debris and other impurities) was removed. 100 μL supernatant and 100 μL DEHs (0.8×10^6–1.2×10^6 cells/mL) were mixed together. The DEHs was treated as internal reference. Total RNA was extracted and Real-time PCR was employed to quantify the amount of virus release.

To analyze the level of virus release, virus release rate of VC group was set to 100%. Then the relative virus release rate can be represented by (release of polysaccharide group/release of VC group)/(replication of polysaccharide group/replication of VC group) × 100%.

2.6. Statistical analysis

$2^{-\Delta\Delta CT}$ (Kenneth & Thomas, 2001) method was used to analysis relative gene expression data. The data of A_{570} value and relative gene expression value were expressed as the mean ± S.D. Duncan's multiple range test was used to analyze the difference among groups with the software SPSS 16.0. *T*-test was used to analyze the difference of the mortality rate. Significant differences were considered as $p < 0.05$.

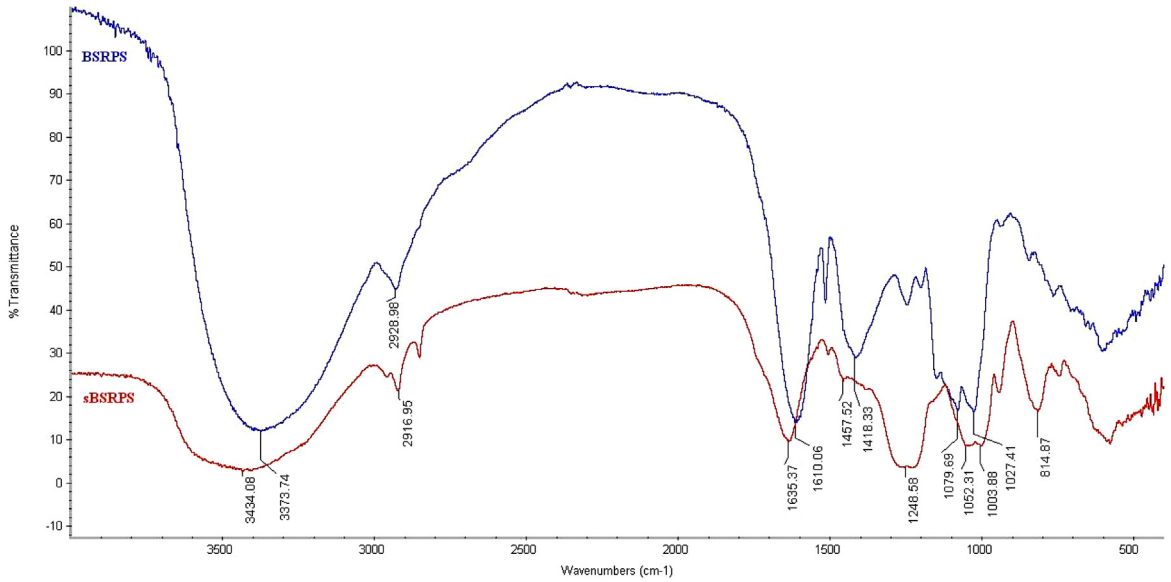

Fig. 1. Infrared spectroscopy of BSRPS and sBSRPS. The bands of 3600–3200 cm^{-1}, 1400 cm^{-1}, 3020–2820 cm^{-1}, 1200–950 cm^{-1} and 1620 cm^{-1} and 1420 cm^{-1} were attributed to the hydroxyl stretching vibration, C—H deformation vibration, C—H stretching vibration, C—O—C and C—O—H stretching vibrations and COO^- stretching vibration, respectively. The bands of 1248 cm^{-1} and 814 cm^{-1} were attributed to the S=O asymmetry stretching vibrations and C—O—S symmetry stretching vibration.

Table 1
The A_{570} values and virus inhibitory rate in antiviral activity against DHAV test on DEHs.

Group	Concentration[A] (μg/mL)	A_{570}	Virus inhibitory rate (%)	Group	Concentration[A] (μg/mL)	A_{570}	Virus inhibitory rate (%)
BSRPS	2000	0.372 ± 0.003^{c}	31.08	sBSRPS	7.813	0.429 ± 0.023^{b}	58.25
	1000	0.365 ± 0.017^{c}	26.35		3.906	0.390 ± 0.015^{b}	39.32
	500	0.405 ± 0.010^{b}	53.38		1.953	0.399 ± 0.027^{b}	43.69
	250	0.356 ± 0.006^{c}	20.27		0.977	0.397 ± 0.022^{b}	42.72
VC		0.326 ± 0.006^{d}		VC		0.309 ± 0.008^{c}	
CC		0.474 ± 0.008^{a}		CC		0.515 ± 0.026^{a}	

Data within a column without the same superscripts (a–d) differ significantly ($p < 0.05$).
[A] The safe concentration determined by pre-experiment of cytotoxicity test.

3. Results

3.1. The infrared spectroscopy characteristic of BSRPS and sBSRPS

The FT-IR spectra of BSRPS and sBSRPS were illustrated in Fig. 1. Specific absorption peaks of polysaccharides (Marcottea, Kegelaera, Sandta, Barbeaub, & Lafleur, 2007; Boulet, Williams, & Doco, 2007) were found in both BSRPS and sBSRPS. The absorption bands in the region of 3600–3200 cm^{-1} were attributed to the hydroxyl stretching vibration. The bands attributed to C—H deformation vibration appeared at about 1400 cm^{-1}. The peaks in the region of 3020–2820 cm^{-1} conformed to C—H stretching vibrations. The absorption peaks in the region of 1200–950 cm^{-1} were related to C—O—C stretching vibrations and C—O—H stretching vibrations. The bands toward around 1620 cm^{-1} and 1420 cm^{-1} were attributed to stretching vibration of COO^-.

Specific absorption peaks of sulfate (Ghosh et al., 2004) were found in sBSRPS. The absorption peaks toward about 1248 cm^{-1} were related to S=O asymmetry stretching vibrations. The peaks attributed to C—O—S symmetry stretching vibration appeared at about 814 cm^{-1}.

3.2. Antiviral activity of BSRPS and sBSRPS in vitro

Table 1 listed the A_{570} values and virus inhibitory rate in anti-DHAV activity test on DEHs. All the A_{570} values of BSRPS and sBSRPS groups were significantly higher than that of VC group. Virus inhibitory rates of sBSRPS group were generally higher than that of BSRPS group (except that of 500 μg/mL).

3.3. Antiviral effect of BSRPS and sBSRPS in vivo

3.3.1. Clinical curative effect

Table 2 listed the deaths of young ducklings of every group. Ducklings of VC group all died within four days after challenging DHAV. No death ducklings in BSRPS and sBSRPS groups were found after 72 h. All ducklings in BC group survived and were fit as a fiddle. Both mortality rates of BSRPS and sBSRPS groups were lower than VC group, and sBSRPS group's mortality rate was significantly lower than VC group.

Table 2
The deaths of young ducklings of every group.

Group	Samples (feather)	Deaths (feather)	Time of death ending (h)	Mortality rate (%)
BSRPS	30	26	72	86.67^{ab}
sBSRPS	30	24	72	80.00^{b}
VC	30	30	–[A]	100.00^{a}
BC	30	0	–[B]	0^{c}

Data within a column without the same superscripts (a–c) differ significantly ($p < 0.05$).
[A] Time of death ending was unable to be analyzed in the test for the 100% death rate.
[B] Time of death ending was unable to be analyzed in the test for no deaths.

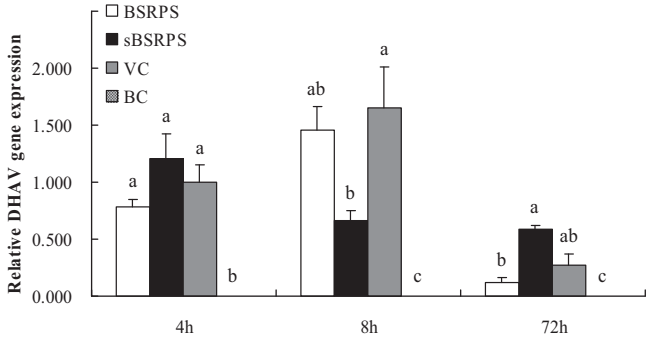

Fig. 2. Quantitative analysis of the dynamics of DHAV gene expression in blood of every group (BC group, VC group, BSRPS group, sBSRPS group) at 4th, 8th and 72nd h after injected virus. Data without the same superscripts (a–c) differ significantly ($p < 0.05$).

3.3.2. Blood DHAV contents of the duck

A quantitative analysis of the DHAV gene expression in blood at the 4th, 8th and 72nd h after injected virus was illustrated in Fig. 2. No DHAV gene expression was found in BC group. DHAV gene expression of VC group in the 4th h after injected virus was set to 1. The dynamic change in VC group indicated the DHAV content first increased and then decreased in vivo of ducklings. The tendency of BSRPS group was similar to VC group. But the relative expressions of BSRPS group were lower than of VC group in each moment separately. The virus contents in sBSRPS group decreased gradually.

At the 4th h after injected DHAV, virus content of BSRPS group was lower than VC group without significant difference and of sBSRPS group was higher than VC group without significant difference. Virus content of BSRPS group was lower than VC group without significant difference at the 8th h after injected DHAV, while virus content of sBSRPS group was significantly lower than VC group. At the 72nd h after injected DHAV, the relative expressions of DHAV gene in blood of BSRPS and sBSRPS groups showed no difference with VC group while sBSRPS group was significantly higher than BSRPS group.

3.4. Mechanism of antiviral activity of BSRPS and sBSRPS in vitro

3.4.1. Influence of BSRPS and sBSRPS to adsorption of DHAV on DEHs

The effect of BSRPS and sBSRPS on adsorption of DHAV evaluated by direct immunofluorescence assay was illustrated in Fig. 3. Dim fluorescence was appeared in CC group (A) and bright fluorescence was appeared in VC group (B), BSRPS group (C, E) and sBSRPS group (D, F). Whether in post-adding drug (C, D) or pre-adding drug (E, F) method, there was no significant difference between the brightness of fluorescence of BSRPS and sBSRPS group and VC group. This

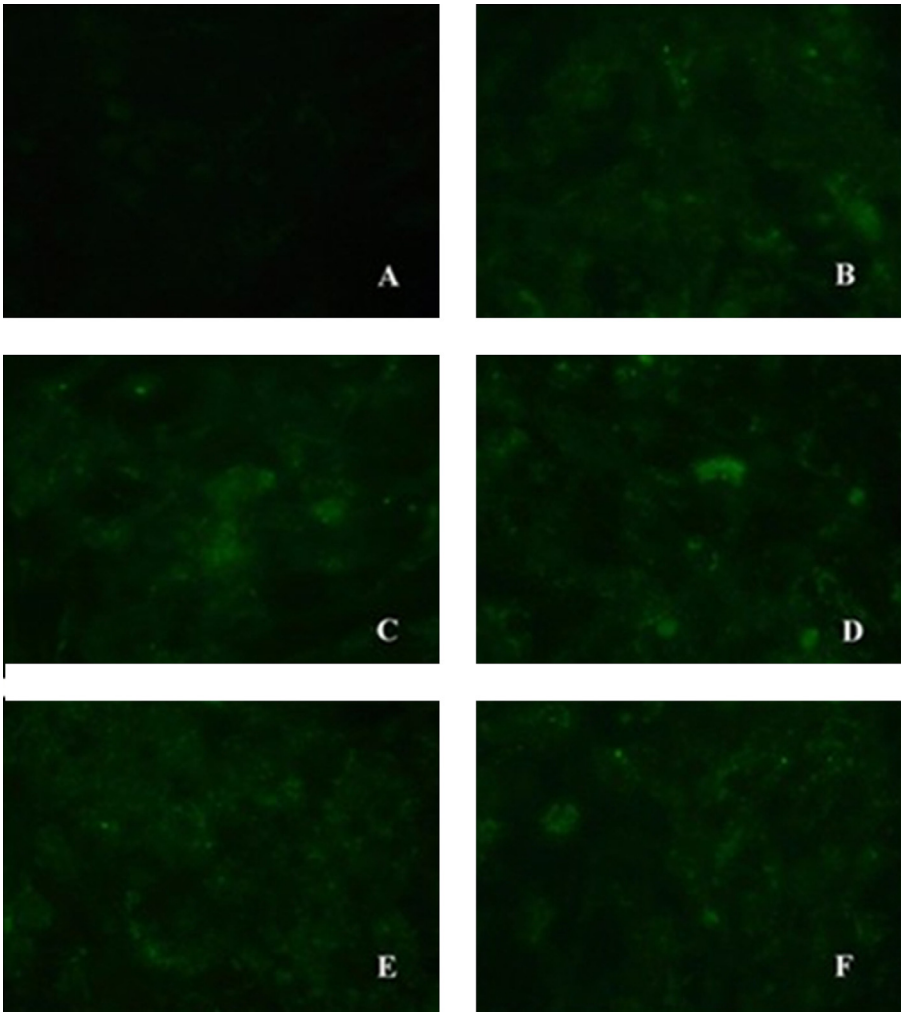

Fig. 3. Direct immunofluroresecence: effect of BSRPS and sBSRPS on DHAV adsorption. DEHs were infected with DHV-1 in the absence (B) or presence of BSRPS (C, E) or sBSRPS (D, F). Drugs were added by post-adding (C, D) and pre-adding (E, F) methods. (A) Cell control group.

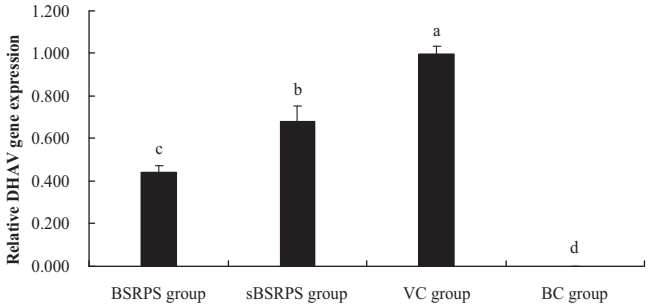

Fig. 4. Effect of BSRPS and sBSRPS on DHAV replication, measured by Real-time PCR assay. Data without the same superscripts (a–d) differ significantly ($p < 0.05$).

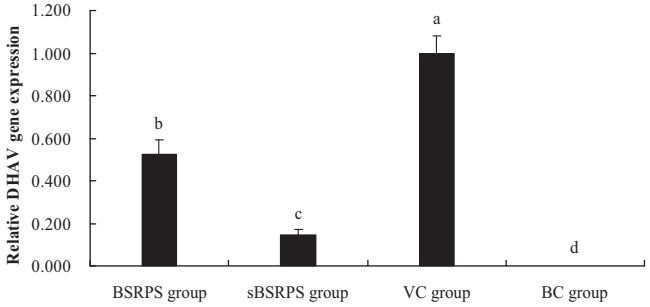

Fig. 5. Effect of BSRPS and sBSRPS on DHAV release in DEHs, measured by Real-time PCR assay. Data without the same superscripts (a–d) differ significantly ($p < 0.05$).

indicated the amounts of DHAV adsorbed on DEHs of BSRPS and sBSRPS groups were similar to VC group in these two methods.

3.4.2. Influence of BSRPS and sBSRPS to replication of DHAV on DEHs

The effect of BSRPS and sBSRPS on replication of DHAV was illustrated in Fig. 4. No DHAV gene expression was found in CC group. DHAV gene expression of VC group in the twenty-fourth hour was set to 1. Relative DHAV gene expressions of BSRPS group and sBSRPS group were 0.442 and 0.679, both were significantly lower than VC group. Relative expression of BSRPS group was significantly lower than sBSRPS group.

3.4.3. Influence of BSRPS and sBSRPS to release of DHAV on DEHs

The effect of BSRPS and sBSRPS on release of DHAV in DEHs measured by Real-time PCR assay was illustrated in Fig. 5. No DHAV gene expression was found in CC group. DHAV gene expression of VC group at the forty-eighth hour was set to 1. Relative expressions of BSRPS group and sBSRPS group were 0.526 and 0.146 while sBSRPS group was significantly lower than BSRPS group. And both were significantly lower than VC group.

The relative release rate of each group was listed in Table 3. BSRPS group's relative release rate was 119.0% and no significant difference compared with VC group. sBSRPS group's relative release rate was 21.6% and was significantly lower than VC group and BSRPS group.

Table 3
The relative release rates (%) of every group.

Item	BSRPS group	sBSRPS group	VC group
Relative virus release rate	119.0 ± 14.4^{a}	21.6 ± 3.6^{b}	100.0 ± 8.3^{a}

Data within a row without the same superscripts (a–b) differ significantly ($p < 0.05$).

4. Discussion

Chlorosulfonic acid–pyridine method which can enhance antiviral activities of polysaccharides is an important one of sulfated (Sun et al., 2009; Wang, Guo, et al., 2010; Liu et al., 2013; Zhang, Hu, et al., 2012). BSRPS was modified with chlorosulfonic acid–pyridine method and sulfate group was introduced successfully (Fig. 1). In the assay of antiviral activity of BSRPS and sBSRPS by MTT method, the higher value of A_{570} is, the higher activity is (Verma et al., 2010). The more viable cells have, the better antiviral activity of polysaccharides shows (Lu, Wang, Hu, Huang, & Wang, 2008). This research discovered that the cytotoxicity of sBSRPS (the maximum safe concentration of sBSRPS was 7.813 μg/mL) was much less than BSRPS (the maximum safe concentration of BSRPS was 2000 μg/mL). In the range of all safe concentration tested, the values of A_{570} of BSRPS and sBSRPS groups were significantly higher than VC group. The maximum virus inhibitory rate of sBSRPS (58.25%) was higher than BSRPS (53.38%). The average virus inhibitory rate of sBSRPS group was higher than that of BSRPS group (Table 1). These instructed that both BSRPS and sBSRPS exhibited the antiviral activity against DHAV in vitro and sBSRPS's antiviral effect was stronger than BSRPS's.

Direct immunofluorescence method is a method to study the position of specific antigen on cells and widely used in antiviral research (Rawlinson, Dwyer, Gibbons, & Cunningham, 1989; Hadziyannis, Sholtis, Schindler, & Lieberman, 1999; Carraro et al., 2007). The stronger brightness of fluorescence is, the more target antigen has (Iwamoto et al., 2000). The brightness of fluorescence of BSRPS and sBSRPS groups had no difference with VC group in post-adding drug and pre-adding drug methods which indicated BSRPS and sBSRPS could not inhibit the adsorption of DHAV on DEHs. The antiviral activity of BSRPS and sBSRPS might have nothing to do with the inhibition of adsorption.

Scholars sequenced the complete genome of DHAV (Kim et al., 2006; Ding & Zhang, 2007). This lays the foundation for molecular detection of DHAV. Yang et al. established one-step real-time Taqman RT-PCR to assay DHAV (Yang, Cheng, Wang, & Xing, 2008). Cheng et al. established reverse transcription-polymerase chain reaction to detect DHAV (Cheng et al., 2009). Real-time PCR with higher specificity, less PCR contamination and higher automation has been widely applied to the diagnosis and detection of DHAV (Luo, Zhang, Xu, Chen, & Liao, 2008). SYBR Green I-Based Real-time PCR was applied to detect the gene expression of DHAV in this paper.

The DHAV gene expression in the forty-eighth hour could not explain the difference of quantity of release was caused by the inhibition of replication or by the difference of quantity of DHAV inside the DEHs. The formula of relative viral release rate (release of polysaccharide group/release of VC group)/(replication of polysaccharide group/replication of VC group) × 100% was introduced to solve this problem. It eliminated the influence of the difference of quantity of DHAV inside the DEHs on the judgment of the release level.

At the twenty-fourth hour, the DHAV gene expression of BSRPS group in replication analysis experiment was 44.2% of VC group while sBSRPS group's was 67.9% of VC group (Fig. 4). There were significant differences among these three groups. This amalgamation suggested BSRPS and sBSRPS demonstrated the inhibition of replication of DHAV in vitro on DEHs and the activity of BSRPS was higher than sBSRPS. At the forty-eighth hour, the DHAV gene expression of BSRPS group in release analysis experiment was 52.6% of VC group while sBSRPS group's was 14.6% of VC group (Fig. 5). And significant differences were demonstrated among these three groups. BSRPS group's relative release rate was similar to VC group, and both significantly higher than that of sBSRPS group (Table 3). These general results signified that BSRPS could inhibit the

replication of DHAV effectively but could not inhibit its release from DEHs and sBSRPS could inhibit both the replication and release. The effect of sBSRPS of inhibition of replication was lower than BSRPS, but the activity of inhibition of release was significantly higher.

Therefore, the anti-DHAV activity of BSRPS on DEHs might result in the inhibition of replication. The antiviral activity of sBSRPS might be mainly owing to the inhibition of replication and release, and the inhibition of release was the main reason. BSRPS could inhibit the replication of virus but showed no capable of release, so it had no shortage of the final total amount of virus. Since although the sBSRPS's capable of inhibition the replication of virus was lower than BSRPS, yet it had very strong capable of inhibition the release, the final total amount of virus of sBSRPS group was significantly less than BSRPS group. That was why the anti-DHAV activity of sBSRPS on DEHs was stronger than BSRPS.

In clinical, DHAV mainly infects young ducklings within three weeks and ducks beyond three weeks can be infected but don't fall ill (Woolcock, 2003). For young ducklings within one week, the morbidity rate and the mortality rate can up to 100%. Hence challenging young ducklings within one week with DHAV (three-day-old) makes sense to the clinical treatment. Treated with BSRPS and sBSRPS, the ending time of death of young ducklings infected DHAV was earlier than VC group. The mortality rates of BSRPS group and sBSRPS group were both less than VC group. And BSRPS group's mortality rate was significantly less than VC group (Table 2). These proved the anti-DHAV activity of BSRPS and sBSRPS in vivo. And the antiviral activity of sBSRPS was stronger than BSRPS in vivo which was similar to that in vitro.

DHAV of the family picornaviridae has a replication cycle between the 6th and 8th h include adsorption, penetration, uncoating, biosynthesis, assembly and release in vivo. At the early period before the 8th h, many defense mechanisms including humoral immunity did not play a fundamental role in anti-DHAV activity; it mainly results in the direct antiviral activity of drugs in the emergency phase. At the fourth hour after injected DHAV, the replication cycle of DHAV had not completed. The BSRPS's inhibition of replication was stronger than sBSRPS, so the blood virus content of BSRPS group was less than sBSRPS group and VC group at this time (Fig. 2). At the eighth hour, at least one replication cycle accomplished. The blood virus content of sBSRPS group was significantly less than BSRPS group and VC group because of the strong inhibition of release of sBSRPS at this time. The blood virus content of BSRPS group was less than VC group but showed no significant difference (Fig. 2). This was owing to BSRPS's significant inhibition of replication to decrease the blood virus content. However, BSRPS could not significantly inhibit the release of DHAV, most of the viruses replicated in the body of young ducklings released, so the blood virus content of SPTS group was similar to VC group. At the seventy-second hour, body's own clean mechanisms including cellular immunity, humoral immunity and non-specific immunity were activated obviously. So the blood virus contents of BSRPS and VC group rapid declined and without significant difference (Fig. 2). sBSRPS group's blood virus content was higher than VC group and significantly higher than BSRPS group (Fig. 2). However, the mortality rates of BSRPS and sBSRPS were lower than VC group, and sBSRPS was lower than BSRPS (Table 2). This suggested that the content of DHAV with limited amount did not influence the deaths in the late course. Obviously, the mechanism of BSRPS and sBSRPS's anti-DHAV activity was not limited to the inhibition of replication and release in vivo. It was worth to further investigate some other relevant mechanisms.

5. Conclusion

BSRPS was modified successfully by chlorosulfonic acid–pyridine method in this experiment. BSRPS and its sulphate sBSRPS exhibited the anti-DHAV activity on DEHs and both of them could reduce the mortality rate caused by DHAV in vivo. The antiviral activity of sBSRPS was significantly stronger than BSRPS. The mechanism is mainly associated with the capable of inhibition of replication and release of BSRPS and sBSRPS. BSRPS's capable of inhibition of replication was significantly higher than sBSRPS's but sBSRPS exhibited stronger capable of inhibition of release. The antiviral activity of BSRPS and sBSRPS might not be associated with the adsorption. Sulfated Bush Sophora Root polysaccharide can be further researched and expected to exploit into a new-type antiviral drug against DHAV.

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