Antiviral Research 98 (2013) 344–351

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Antiviral activity of sulfated *Chuanminshen violaceum* polysaccharide against duck enteritis virus *in vitro*

Xu Song [a,1], Zhongqiong Yin [a,b,1,*], Li Li [a,1], Anchun Cheng [a,b], Renyong Jia [a,b], Jiao Xu [a], Yin Wang [a], Xueping Yao [a], Cheng Lv [a], Xinghong Zhao [a]

[a] College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, China
[b] Institute of Prevention Veterinary Medicine, Sichuan Agricultural University, Chengdu 611103, China

ARTICLE INFO

Article history:
Received 16 December 2012
Revised 2 March 2013
Accepted 4 March 2013
Available online 21 March 2013

Keywords:
Sulfated modification
Chuanminshen violaceum polysaccharide
Heparan sulfate
Antiviral activity
Duck enteritis virus

ABSTRACT

Duck enteritis virus (DEV) of the family *Herpesviridae* is one of the main diseases in waterfowl. Despite the wide use of vaccines to control the disease, infection with the virus cannot be completely prevented. Therefore, antiviral agents against DEV should be developed. This study presents a novel sulfated polysaccharide from *Chuanminshen violaceum* (sCVPS), which exhibits significant antiviral activity against DEV with 50% inhibitory concentrations (IC_{50}) ranging from 77.12 μg/mL to 104.81 μg/mL. sCVPS is more effective than heparan sulfate (HS, as a positive control) with IC_{50} = 132.61 μg/mL. sCVPS and HS inhibit viral activity by preventing virus adsorption with IC_{50} values ranging from 82.83 μg/mL to 109.28 μg/mL for sCVPS and 150.22 μg/mL for HS. Direct immunofluorescence assay and transmission electron microscopy demonstrated that the mechanism of action was the interference with virus adsorption. The amount of inhibited virus during adsorption was quantified using fluorescent quantitative polymerase chain reaction, which revealed that both sCVPS and HS can significantly reduce all viruses attached to cells. sCVPS also prevented the cell-to-cell spread of DEV. These results indicated that sCVPSs perform more effectively than does HS as antiviral agents against DEV and can be further examined for potential effects as an alternative control measure for DEV infection.

1. Introduction

The *Herpesviridae* are a large family of double-stranded DNA viruses that cause serious diseases in humans and animals (Siakallis et al., 2009). Duck enteritis virus (DEV), a member of the herpesvirus, can cause an acute infectious viral disease called duck virus enteritis (DVE) or duck plague in ducks, geese, swans, and other waterfowl species (Mondal et al., 2010; Qi et al., 2009). DEV infection can result in high mortality, carcass condemnation, and reduced egg production or hatchability (Wang et al., 2011a; Wu et al., 2012a). Despite the use of attenuated vaccines that can provide protection against DEV, infection is not prevented completely (Li et al., 2009; Mondal et al., 2010; Wang and Osterrieder, 2011; Wang et al., 2011a). Moreover, no antiviral agents against DEV are available.

The antiviral activity of sulfated polysaccharides was first reported in 1958 by Gerber (Gerber et al., 1958), who found that polysaccharides extracted from *Gelidium cartilagenium* prevented influenza B and mumps virus infection in embryonated eggs. Many sulfated polysaccharides have been found to possess a broad spectrum of antiviral activity since the report (Han et al., 2010). The inhibitory effect of sulfated polysaccharides can potentially block early stages of viral life, including initial attachment to the target cell and viral entry (Damonte et al., 2004; Harden et al., 2009). Therefore, sulfated polysaccharides exhibit an inhibitory activity mainly by interaction with viral envelope proteins involved in virus infection of susceptible cells. In DEV, glycoprotein C significantly affects virus adsorption and transmission, binding to heparan sulfate proteoglycans present on the cell surface, leading to initial attachment (Lian et al., 2010). DEV entry is mediated by the coordinated effects of glycoprotein B, D, and H/L with cell receptors, resulting in fusion and penetration (Heldwein and Krummenacher, 2008; Li et al., 2009; Spear and Longnecker, 2003). These glycoproteins may act as candidate target receptors in anti-DEV drug design.

Chuanminshen violaceum is a traditional Chinese medicinal herb used as a tonic. *C. violaceum* polysaccharides (CVPSs) mainly constitute (almost 28%) *C. violaceum* (Lei and Zhang, 2012). CVPS is composed of D-carubinose and D-glucose with a ratio of 1:16.2 (Zhang et al., 2007). The weight average molecular weight and the number average molecular weight of CVPS are 9.7632×10^5 Da and 5.2270×10^4 Da, respectively (Zhang et al., 2007). CVPS can strengthen specific and nonspecific humoral immunity in mice and exhibits an anti-mutagenic effect (Li and Shao, 1996; Zhang et al., 2007).

* Corresponding author at: College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, China. Tel.: +86 835 2885614; fax: +86 835 2885302.
E-mail address: yinzhongq@163.com (Z. Yin).
[1] These authors contribute equally to this work and should be considered as the first author.

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.03.012

Sulfated modification has been widely used to improve polysaccharide structure and enhance antiviral activity (Huang et al., 2008a; Lu et al., 2008; Wang et al., 2010a; Zhao et al., 2011). Moreover, some polysaccharides that originally showed no antiviral activity exhibited antiviral activity after sulfated modification (Huang et al., 2008a). In this paper, we report on the sulfated modification of CVPSs by the chlorosulfonic acid-pyridine method and the characterization of its antiviral efficacy against DEV. This investigation aims to provide a new anti-herpesvirus drug candidate with development potential and a new alternative control measure for DEV infection.

2. Materials and methods

2.1. Sulfated modification of CVPS

CVPS was extracted and purified in our laboratory (Song et al., 2013). Sulfated CVPS (sCVPS) was prepared by the chlorosulfonic acid-pyridine method (Guo et al., 2009b). Chlorosulfonic acid was added dropwise to pyridine (1:4, 1:6, and 1:8) in an ice-water bath, with stirring. Polysaccharides (300 mg) dispersed in dry *N,N*-dimethylformamide (20 mL) were then added to the mixtures and stirred in a water bath at 60 °C for 2 h. The solution was subsequently neutralized with NaOH, dialyzed, and lyophilized to yield sulfated CVPS. The sulfur content of three sCVPSs was measured by the barium chloride–gelatin method (Dodgson and Price, 1962).

2.2. Cell culture and virus infection

A duck embryo fibroblast (DEF) was grown in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) bovine calf serum (Gibco®), 100 U/mL penicillin, and 100 μg/mL streptomycin. For the maintenance medium (MM), serum concentration was reduced to 2%.

DEV (CH virulent strain) was provided by the Institute of Prevention Veterinary Medicine (Chengdu, China). Virus stocks were propagated in DEF and prepared as described in a previous report (Guo et al., 2009a).

2.3. Cytotoxicity assays

The cytotoxicity of CVPS, sCVPS, and HS (H7640; sulfur content: 5–7%; Sigma–Aldrich, USA) was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich, USA) method (Wang et al., 2011b). Thereafter, 2.5×10^4 DEF cells were added to each well of 96-well plates and incubated at 37 °C for 24 h. The growth medium was withdrawn; MM containing twofold dilutions of compounds was added in triplicate. After 72 h of incubation at 37 °C, 10 μL of PBS containing MTT (final concentration: 0.5 mg/mL) was added to each well. Plates were reincubated at 37 °C for 4 h, the supernate in each well was aspirated, and 200 μL of dimethyl sulfoxide was added to solubilize the formazan crystals. After vigorous shaking, absorbance values were measured in a microplate reader (Bio-Rad, USA) at 570 nm. The 50% cytotoxic concentration (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.4. Antiviral activity of sCVPS and HS

Antiviral activity was evaluated by plaque reduction assay (Talarico et al., 2005). The DEF monolayer in 24-well plates was infected with about 50 plaque-forming units (PFU)/well with or without various concentrations of test polysaccharides. After adsorption for 1 h at 37 °C, the medium was replaced with MM containing 1% methylcellulose and a corresponding dose of each compound. After incubation for 72 h at 37 °C, the cells were stained with 0.1% crystal violet in 20% methanol. Plaques were counted, and the 50% inhibitory concentration (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50% according to the Reed–Muench method (Reed and Muench, 1938).

2.5. Inhibitory action assays

2.5.1. Pretreatment assay

Dilutions of test polysaccharides were added to each well of 24-well plates containing a DEF monolayer, and the plates were incubated at 37 °C for 1 h. The compounds were removed, and the monolayer was washed thrice with PBS (pH = 7.4). Virus suspensions (50 PFU/well) were then added to each well. After incubation at 37 °C for 1 h, the cells were rinsed thrice with PBS and overlaid with MM containing 1% methylcellulose. The plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.2. Virus adsorption assay

A DEF monolayer grown in 24-well plates was infected with about 50 PFU/well with or without various concentrations of test polysaccharides. After incubation at 4 °C for 1 h, the monolayer was washed thrice with cold PBS to remove unadsorbed viruses and then overlaid with MM containing 1% methylcellulose. Plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.3. Virus penetration assay

A DEF monolayer grown in 24-well plates was infected with about 50 PFU/well and incubated at 4 °C for 1 h. Virus inocula were aspirated and washed twice to remove unadsorbed viruses. Dilutions of test polysaccharides were added to each well, and plates were incubated at 37 °C for 1 h to allow penetration. After removal of compounds, the monolayer was rinsed with a citrate buffer (pH 3.0) to inactivate virions that had not penetrated the cells. MM containing 1% methylcellulose was then added to each well, and plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.4. Virus replication assay

A DEF monolayer grown in 24-well plates was infected with about 50 PFU/well and incubated at 37 °C for 2 h to allow adsorption and penetration. MM containing 1% methylcellulose and dilutions of test polysaccharides was then added to each well, and plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.5. Virus inactivation assay

MM containing dilutions of test polysaccharides was incubated with an equal volume of virus suspension (5×10^5 PFU) at 37 °C for 1 h. The solution was then diluted 10^4 times, and the titer of residual infectious virus was quantified by plaque assay.

2.6. Plaque size assay

A DEF monolayer grown in 6-well plates was infected with about 100 PFU/well. After incubation at 37 °C for 2 h to allow penetration, the inocula were removed. MM containing 1% methylcellulose and $sCVPS_{1.37}$ (50, 100, and 200 μg/mL) was then added, and plates were incubated at 37 °C for 72 h. The plaque size was calculated using Nikon Imaging Software-Elements. The average plaque

size was obtained by measuring 20 plaques from each group (Antoine et al., 2012).

2.7. Direct immunofluorescence assay

A DEF monolayer grown in cover slips was infected with DEV (multiplicity of infection [MOI] = 1.0) with or without $sCVPS_{1.37}$ (200 μg/mL). After 48 h of infection, the monolayer was washed with cold PBS and fixed in methanol at −20 °C for 15 min for cytoplasmic immunofluorescence. The monolayer was permeabilized using 0.5% (v/v) Triton X-100 in PBS for 5 min, washed thrice with PBS, and incubated with fluorescein isothiocyanate-labeled rabbit anti-DEV (bs-1672R-FITC; Beijing Biosynthesis Biotechnology) at 37 °C for 1 h. Cells were finally washed and directly observed using a fluorescence microscope (Eclipse 80i, Nikon, Japan) (Xu et al., 2012).

2.8. Transmission electron microscopy assay

A DEF monolayer grown in culture flasks was infected with DEV (MOI = 1.0) with or without $sCVPS_{1.37}$ (200 μg/mL). The monolayer was washed and scraped with a cell scraper (Nunc, USA) 48 h after infection. Cells were then collected by centrifugation at 1000 rpm for 5 min and fixed in 2.5% glutaraldehyde at 4 °C for 2 h. After several buffer washes, fixed samples were post-fixed in 1% osmium tetroxide at 4 °C for 2 h. Samples were dehydrated in a graded acetone series prior to infiltration and embedding. Ultrathin (100 nm, Leica UC6) longitudinal sections were cut, stained with uranyl acetate and lead citrate, and examined under a Tecnai G^2 F20 electron microscope (FEI, USA).

2.9. Fluorescent quantitative polymerase chain reaction assay

A DEF monolayer grown in 6-well plates was infected with DEV (MOI = 0.1) with or without sCVPS (400, 200, and 100 μg/mL) and HS (100 μg/mL). After incubation at 4 °C for 1 h, the monolayer was washed thrice with cold PBS to remove unadsorbed viruses. Total DNA was extracted from cells by using DNAiso Reagent (D305; Takara, China) according to the manufacturer's instructions. The primers and probe based on the UL30 gene of DEV (CH virulent strain; Genbank: JQ647509) were designed using Primer Premier Software (version 5.0; Premier Biosoft International, Palo Alto, CA). The forward and reverse primers were 5′-CCCAAACACGAAACATGC-3′ (positions: 61418–61435) and 5′-TGTCCGGTTACAATATCGTT-3′ (positions: 61522–61541), respectively. A 22 bp probe, 5′-FAM-CTCCTTTGTTCATCGCCCCGTA-TAMRA-3′ (positions: 61436–61457), was also selected. A 124 bp fragment was amplified with the primers and cloned in pMD 19-T Vector (D102; Takara, China), used as a standard DEV DNA. The recombinant plasmid was subsequently quantified by measuring the optical density at 260 nm, and corresponding copy numbers were calculated using the equations described previously (Wang et al., 2002). Serial 10-fold dilutions of standard DEV DNA from 1×10^1 to 1×10^6 copies/reaction tube were prepared to construct a standard curve by using *Premix* Ex Taq™(Probe qPCR) (DRR390; Takara, China). Fluorescent quantitative polymerase chain reaction (FQ–PCR) was performed at 95.0 °C for 30 s, 95.0 °C for 5 s (40 cycles), and 57.5 °C for 30 s by using a Bio-Rad CFX96 Touch™ real-time PCR detection system.

3. Results

3.1. Characterization of sCVPS

Sulfated derivatives of CVPS were prepared as described above. The sulfur contents (S%) of the three sCVPSs (14.55%, 11.71%, and 9.48%) were determined by the barium chloride–gelatin method (Dodgson and Price, 1962). The degrees of sulfation (DS) of $sCVPS_{1.37}$, $sCVPS_{0.95}$, and $sCVPS_{0.69}$ were 1.37, 0.95, and 0.69, respectively, as determined by the following equation: $DS = (1.62 \times S\%)/(32–1.02 \times S\%)$ (Guo et al., 2009b). The average molecular weights of $sCVPS_{1.37}$, $sCVPS_{0.95}$, and $sCVPS_{0.69}$ were 1.20×10^5 Da, 2.54×10^5 Da, and 3.81×10^5 Da, respectively, as determined by gel permeation chromatography. The sCVPSs were homogeneous, as shown by agarose gel electrophoresis. The monosaccharide composition slightly changed, as determined by gas chromatography–mass spectrometry analysis.

3.2. Antiviral activity of sCVPS and HS

The antiviral activities of sCVPS and HS, which were evaluated for their ability to inhibit DEV plaque formation in DEF, are summarized in Table 1. Test polysaccharides exhibited no cytotoxicity at the maximum concentration tested (2000 μg/mL). CVPS initially exhibited no anti-DEV activity; however, sulfated derivatives exhibited activity with varying levels of effectiveness according to the DS. Among the three sCVPSs, $sCVPS_{1.37}$ exhibited the highest activity (IC_{50} = 77.12 μg/mL). $sCVPS_{0.95}$ exhibited a higher degree of inhibitory action (IC_{50} = 94.04 μg/mL) than did $sCVPS_{0.69}$ (IC_{50} = 104.81 μg/mL). sCVPS also showed higher anti-DEV activity than did HS (IC_{50} = 132.61 μg/mL). Moreover, the anti-DEV activities of sCVPS and HS were specific in terms of selectivity index.

3.3. Inhibitory action studies

Five tests were designed to analyze the influence of test polysaccharide treatment during various phases of the viral life cycle. Pretreatment assay that tests polysaccharides with DEF monolayers prior to virus infection detected no activity (Table 2), suggesting that the test polysaccharides cannot bind to virus receptors on the cell surface involved in the initial virus attachment. However, when added to the monolayer during virus adsorption, sCVPS and HS showed markedly enhanced inhibitory effects with IC_{50} values ranging from 82.83 μg/mL to 109.28 μg/mL for sCVPS and an IC_{50} of 150.22 μg/mL for HS (Table 2). In the penetration experiment, the virus was incubated with the DEF monolayer at 4 °C for 1 h to allow adsorption prior to addition of test polysaccharides. Compound dilutions were then added during a 1 h penetration step at 37 °C. sCVPS and HS exhibited very poor inhibition of virus penetration at the maximum concentration tested (2000 μg/mL) (data not shown in Table 2). After penetration into host cells, the virus began its replication by using host resources. Experiments were thus conducted to measure the effect of test polysaccharides on virus replication. No compound exhibited an inhibitory effect during virus replication (Table 2). In the antiviral trials of test polysaccharides designed according to the viral life cycle, compounds were at times directly incubated with the virus during infection.

Table 1
Antiviral activity of sCVPS, HS and CVPS against duck enteritis virus in duck embryo fibroblast.

Compounds	CC_{50} (μg/mL)[a]	IC_{50} (μg/mL)[b]	SI[c]
$sCVPS_{1.37}$	>2000	77.12 ± 2.36	>26
$sCVPS_{0.95}$	>2000	94.04 ± 2.30	>21
$sCVPS_{0.69}$	>2000	104.81 ± 1.75	>19
HS	>2000	132.61 ± 3.65	>15
CVPS	>2000	–	–

[a] Cytotoxic concentration 50% (CC_{50}), concentration required to reduce DEF viability by 50%, was measured by MTT method.
[b] Inhibition concentration 50% (IC_{50}): concentration required to reduce virus plaques by 50%.
[c] SI: Selectivity index is defined as the ratio of CC_{50} to IC_{50} ($SI = CC_{50}/IC_{50}$).

Table 2
Effect of sCVPS, HS and CVPS on pretreatment, adsorption, penetration, replication and inactivation assay against duck enteritis virus in duck embryo fibroblast.

Compounds	Pretreatment [a]	Adsorption [a]	Penetration [a]	Replication [a]	Inactivation [a]
$sCVPS_{1.37}$	–	82.83 ± 2.16	–	–	–
$sCVPS_{0.95}$	–	101.14 ± 3.83	–	–	–
$sCVPS_{0.69}$	–	109.28 ± 1.36	–	–	–
HS	–	150.22 ± 3.00	–	–	–
CVPS	–	–	–	–	–

[a] Values shown represent the concentration required to inhibit plaque formation by 50% and are given in units of μg/mL.

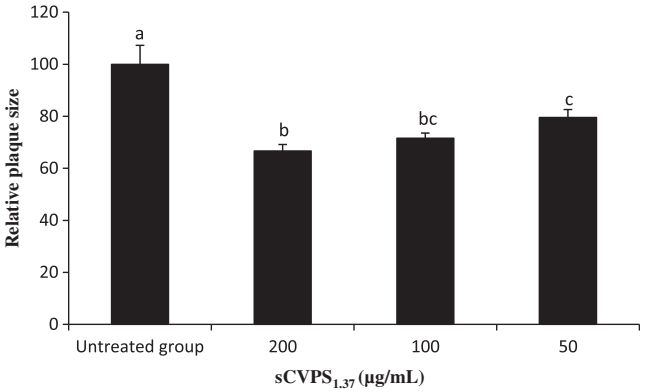

Fig. 1. The relative plaque size of untreated and sCVPS-treatment groups. DEF monolayer grown in 6-well plates was infected with about 100 PFU/well. After virus penetration, MM containing 1% methylcellulose and $sCVPS_{1.37}$ (50, 100, 200 μg/mL) was then added and plates were incubated at 37 °C for 72 h. The size of Plaques was determined and values were obtained by three independent experiments. The different letters on a column differ significantly ($P < 0.05$).

To test whether the observed antiviral effects of sCVPS were related to its inactivation, concentrated DEVs were incubated with dilutions of test compounds *in vitro*. Meanwhile, inactivation was measured, but no activity was detected (Table 2).

3.4. Plaque size study

A study of the mode of action revealed that the sCVPS exerted no effect on virus replication after virus penetration into host cells. However, mature progeny virions were released into extracellular spaces after complete virus replication (Fig. 3B) and subsequently spread laterally between adjacent cells. Consequently, whether sCVPS can inhibit progeny virions from infected adjacent cells is determined; thus, sCVPS was present during plaque formation. Plaque size in the sCVPS-treated groups was significantly reduced ($P < 0.05$) by almost 40% of that in the untreated group (Fig. 1). A significant difference in plaque size was indicated between the sCVPS group with a concentration of 200 μg/mL and the sCVPS group with a concentration of 50 μg/mL.

3.5. Direct immunofluorescence study

Direct immunofluorescence assay measured the effect of sCVPS on viral antigen expression. Cells were infected with DEV (MOI = 1.0) with or without $sCVPS_{1.37}$ (200 μg/mL). Viral antigen expression was observed by immunofluorescence assay 24 h after infection. High fluorescence of viral antigens was detected in DEV-infected cells without sCVPS treatment (Fig. 2A). By contrast, virus antigen expression in DEV-infected cells with sCVPS treatment drastically decreased, and very low fluorescence was detected in cells (Fig. 2B). Non-fluorescence was also observed in non-infected cells (Fig. 2C).

3.6. Transmission electron microscopy study

Transmission electron microscopy (TEM) was used to describe the state of virus propagation in DEV-infected cells. Cells were infected with DEV (MOI = 1.0) with or without $sCVPS_{1.37}$ (200 μg/mL). The cells were processed for TEM 48 h after infection. In DEV-infected cells without sCVPS treatment, many DEV particles (approximately 150 nm in diameter; Guo et al., 2009a) were found in vacuoles in the cytoplasm (Fig. 3B and C), and mature progeny viruses remained unreleased into extracellular spaces (Fig. 3B). Vacuolar degeneration caused by DEV infection was also observed (Fig. 3C). In sCVPS-treated cells, a small number of DEV particles were found in both intracellular and extracellular spaces (Fig. 3D and 3E), and the cells were shown to exhibit a normal structure. No virus particles were observed in uninfected cells (Fig. 3A).

3.7. Fluorescent quantitative polymerase chain reaction study

FQ–PCR was employed to quantify the amount of virus inhibited by sCVPS and HS during adsorption. Cells in 6-well plates were infected with DEV (MOI = 0.1) with or without sCVPS and HS. After adsorption at 4 °C for 1 h, the total DNA in each well was extracted. The standard curve with detection ranging from 1×10^1 to 1×10^6 copies/reaction ($R^2 = 1.00$; data not shown) indicates that both sCVPS and HS can significantly ($P < 0.05$) reduce all viruses attached to cells (Fig. 4). sCVPS showed higher activity than did HS ($P < 0.05$), and $sCVPS_{1.37}$ showed the highest activity among all test polysaccharides ($P < 0.05$). Significant differences in the three sCVPS groups were observed among the test concentrations (400, 200, and 100 μg/mL).

4. Discussion

A number of sulfated naturally and chemically synthesized polysaccharides have shown antiviral activity against herpesviruses, particularly against the herpes simplex virus (HSV-1 and HSV-2) (Pujol et al., 2007; Wijesekara et al., 2011; Yasuhara-Bell and Lu, 2010). In the present study, sulfated modification of CVPS was successfully performed by the chlorosulfonic acid–pyridine method. This study is the first to report on the anti-DEV activity of CVPS derivatives (Table 1): sCVPS was a potent inhibitor of DEV in DEF ($IC_{50} < 105$ μg/mL), with the selectivity index (>19) evaluated by plaque reduction assay. These results support the view that sulfated modification is one of the most effective approaches to improving the antiviral activity of polysaccharides. Another technique in polysaccharide modification to enhance antiviral activity has been reported: phosphorylated modification of *Polygonatum cyrtonema* polysaccharide can increase its anti-herpetic activity (Liu et al., 2011).

Compared with the potency of other sulfated polysaccharides against herpesvirus, the inhibitory activity of sCVPS was weaker given that the IC_{50} values of many sulfated polysaccharides were lower than 50 μg/mL, whereas that of sCVPS was 77.12 μg/mL

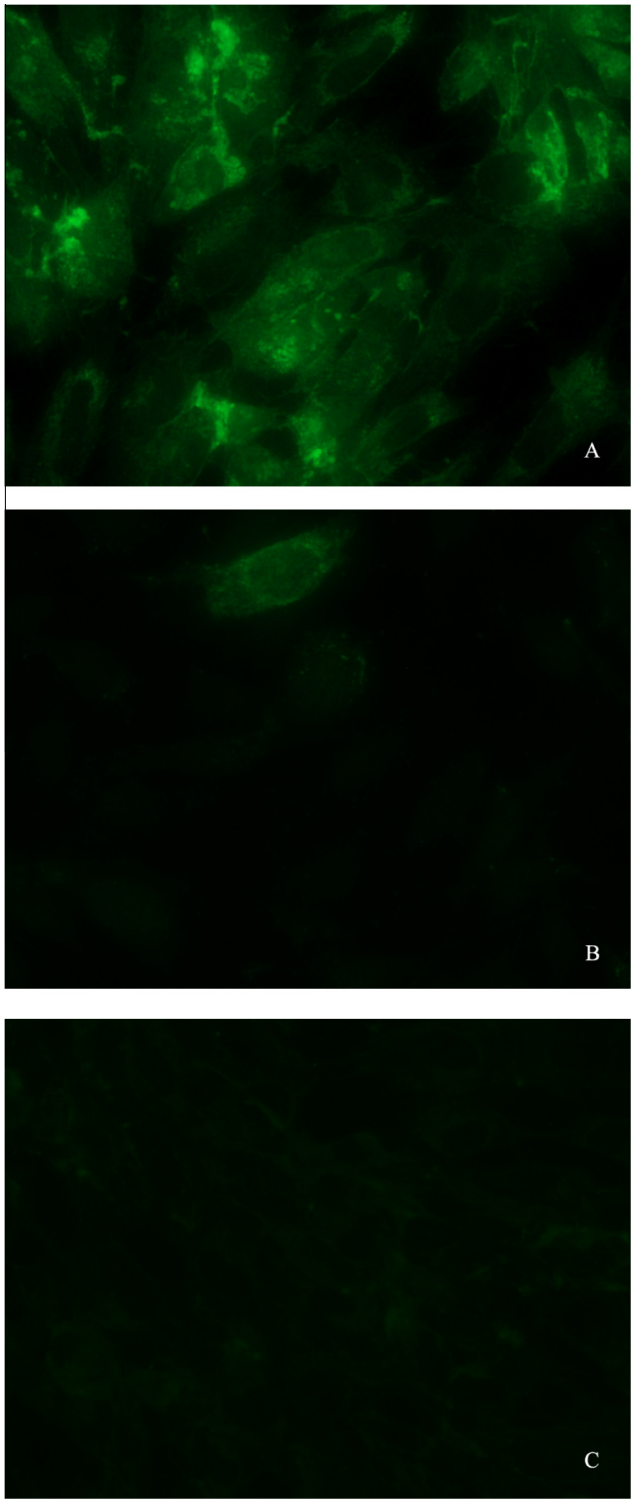

Fig. 2. Anti-DEV effect of sCVPS by immunofluorescence assay. Cells were infected with DEV (MOI = 1.0) in the presence (B) or absence (A) of $sCVPS_{1.37}$ (200 μg/mL). At 24 h post-infection, viral antigen expression was observed by immunofluorescence assay. (C) Mock infected cells.

(Table 1) (Harden et al., 2009). Similar results were observed in sulfated agarans from *Bostrychia montagnei*, *Calendula officinalis*, and *Georgiella confluens*: the IC_{50} values against HSV-1 and HSV-2 were higher than 50 μg/mL when the sulfur content ranged from 6.4% to 12.6% (Damonte et al., 2004). Spirulan from *Spirulina platensis* (sulfur content = 3.24%) was also found to exhibit weak inhibitory activity, with an IC_{50} value of 155 μg/mL against HSV-1 (Hayashi et al., 1996). Thus, differences in efficacy were caused mainly by sulfur content and the characteristics of the polysaccharides themselves, including sugar composition, molecular weight, and molecular conformation (Harden et al., 2009).

Generally, the antiviral activity of sulfated polysaccharides increases with the DS within a certain range (Damonte et al., 2004). In some cases, the highest DS is not accompanied by the highest antiviral activity. In the present study, sCVPS with a higher DS exhibited higher anti-DEV activity ($sCVPS_{1.37}$ > $sCVPS_{0.95}$ > $sCVPS_{0.69}$), suggesting that the sulfation of CVPS was within an optimal scope, leading to an inhibitory effect that accompanies DS.

HS proteoglycans are expressed by most mammalian cells, frequently present in cell membranes, where they act as cell surface receptors during initial virus attachment to host cells. These viruses include the herpes virus, dengue virus, adeno-associated virus, and human immunodeficiency virus type 1 (Pourianfar et al., 2012; Rabenstein, 2002; Waarts et al., 2005). Therefore, HS and its analogs exhibit a broad spectrum of antiviral activity because of their significant effect on virus infection (Lee et al., 2006; Nyberg et al., 2004; Pourianfar et al., 2012). The first to evaluate the antiviral activity of HS against DEV, the present study found that HS effectively inhibited DEV with an IC_{50} of 132.61 μg/mL, but such efficacy was lower than that of sCVPS (Tables 1 and 2). This difference in efficacy between HS and sCVPS was due to the difference in sulfur contents; the sulfur content of HS was about 7% lower than that of sCVPS (9.48–14.55%).

Binding to the cell surface receptor is a critical step in cell invasion by a virus. In antiviral drug development, prevention of viral infection at this early stage is considered less toxic than chemotherapy (Liu and Thorp, 2002). Previous studies showed that the antiviral activity of sulfated polysaccharides primarily resulted from interference with the initial adsorption of the virus to the host cells (Damonte et al., 2004). This finding is consistent with the results of the present study. The antiviral activities of sCVPS and HS were attributed to the inhibition of virus adsorption measured by plaque reduction assay (Table 2) and further confirmed by direct immunofluorescence assay (Fig. 2) as well as TEM (Fig. 3). This study also established a new FQ–PCR method to detect DEV by which sCVPS and HS significantly reduced the amount of virus during adsorption in a dose-dependent manner with MOI of 0.1 (Fig 4). Thus, the prevention efficacy for binding DEV–DEF by sCVPS and HS was independent of the input viral count.

The mechanism of action is mediated by the interactions of anionic charges in sulfated polysaccharides with positively charged domains of viral envelope glycoproteins. These interactions shield the functional domain involved in virus attachment to cell surface receptors (Damonte et al., 2004; Harden et al., 2009; Witvrouw and De Clercq, 1997). The envelope glycoprotein C of DEV is nonessential for propagation but responsible for virus binding (Li et al., 2009; Wang and Osterrieder, 2011). Therefore, sCVPS was believed to act on glycoprotein C and competitively inhibit the binding of DEV–HS proteoglycans on the cell surface.

After binding to host cells, a virus immediately penetrates by the fusion of the virion envelope with a cell plasma membrane. This process requires the interaction of several envelope glycoproteins with cell surface receptors. No sCVPS and HS can inhibit 50% DEV penetration into DEF at the maximum concentration (Table 2), which may be due to the ineffective interaction of sulfated polysaccharides with envelope glycoproteins for DEV fusion. Although no inhibition of virus replication was detected, sCVPS prevented the cell-to-cell spread of DEV. This observation was reflected by the plaque size (Fig. 1) and attributed to the interference with virus adsorption. Inhibition of DEV infection by sCVPS prevented both the entry and the spread of DEV.

Inactivation is an important antiviral property of sulfated polysaccharides because of its association with increased anti-her-

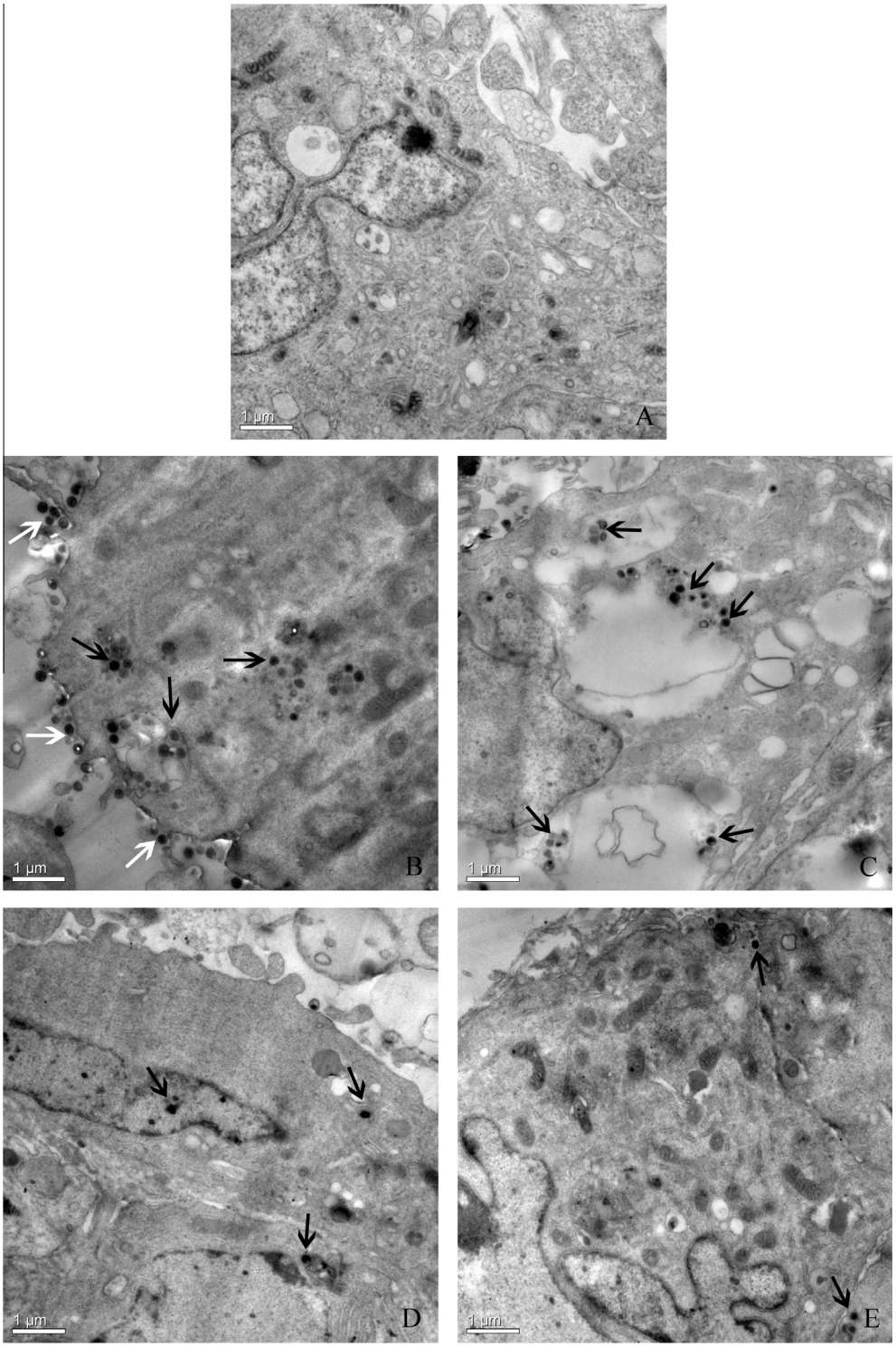

Fig. 3. Anti-DEV effect of sCVPS by transmission electron microscopy assay in DEF. DEF monolayers were infected with DEV (MOI = 1.0) in the presence (D and E) or absence (B and C) of $sCVPS_{1.37}$ (200 μg/mL). At 48 h post-infection, virions in cells were examined by transmission electron microscopy. Without sCVPS-treated, many DEV particles were observed in DEF (B and C, denoted by arrowhead) and the mature progeny viruses had yet been released into extracellular spaces (B, denoted by white arrowhead). Vacuolar degeneration caused by DEV infection was also observed (C). In contrast, few virions were found in sCVPS-treated cells (D and E, denoted by arrowhead) and the cells were shown to exhibit a normal structure. (A) Mock infected cells.

pesvirus activity *in vivo* (Carlucci et al., 2004). The mechanism of virus inactivation by sulfated polysaccharides involves the formation of a stable virion-sulfated polysaccharide complex, causing the occupancy of sites on the viral envelop required for viral infection (Harden et al., 2009). The study on the mode of inhibitory action indicated that sCVPS and HS mainly inhibited viral activity by preventing virus adsorption but did not inactivate the virus inocula by pre-incubation with sCVPS and HS. This finding might have been due to the reversibility of the complex sCVPS–DEV or HS–DEV. When the virus inocula containing sCVPS and HS were diluted 10^4-times for plaque measurement, the number of sulfated polysaccharides decreased sharply, leading to undetected inactivation. Similar

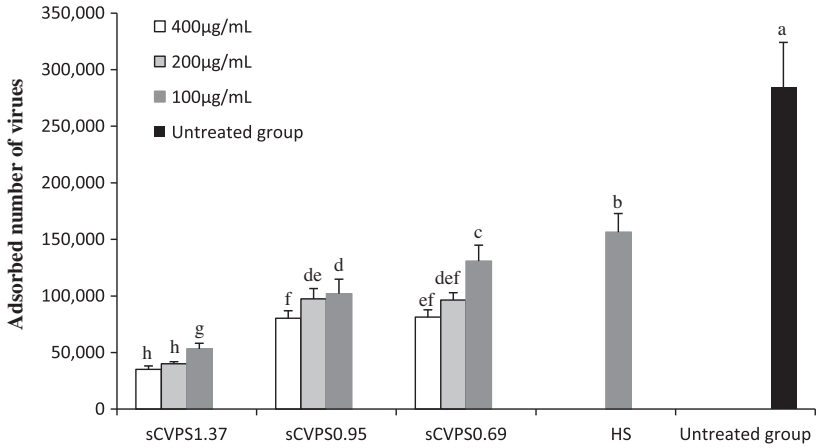

Fig. 4. Anti-DEV effect of sCVPS and HS by fluorescent quantitative polymerase chain reaction assay. Cells in 6-well plates were infected with DEV (MOI = 0.1) in the presence or absence of sCVPS (400, 200 and 100 μg/mL) and HS (100 μg/mL). After 1 h adsorption at 4 °C, total adsorped number of viruses was quantified according to standard curve with detection ranged from 1×10^1 to 1×10^6 copies/reaction (R^2 = 1.00, data not shown). The different letters on a column differ significantly ($P < 0.05$).

results were reported for dextran sulfate and other sulfated polysaccharides (Harden et al., 2009).

DVE is one of the most widespread and devastating waterfowl diseases, causing severe economic loss in the waterfowl industry (Wu et al., 2012b). DVE can be transmitted by direct contact with infected birds as well as indirect contact with a contaminated environment; thus, water seems to be a natural channel of DEV transmission. (Saif et al., 2008). Adding sCVPS to drinking water may be a primary and simple method of preventing DEV infections in waterfowl. *In vivo* sCVPS protection from DEV infections through various administration routes remains under investigation. Sulfated polysaccharides also exhibited numerous advantages over other classes of antiviral drugs, such as relatively low production costs, a broad spectrum of antiviral properties, and low cytotoxicity (Wijesekara et al., 2011). Moreover, sulfated modification of polysaccharides can increase immunity-enhancing activity (Ma et al., 2010; Huang et al., 2008b; Guo et al., 2009b; Wang et al., 2010b). Given its abundant sources, potent anti-DEV activity, and predicted immunity-enhancing activity, sCVPS exhibits potential for the control of DEV infection in ducks.

5. Conclusions

Sulfated modification of CVPS was successfully conducted. This study was the first to demonstrate that sCVPS inhibits DEV and performs more efficiently than does heparan sulfate (positive control). The mechanism of action was related to the inhibition of virus adsorption. With the promising *in vitro* antiviral properties reported in this study, sCVPS exhibits potential for further antiviral research.

Acknowledgements

This study was supported by the National Science and Technology Program in Rural Areas during the 12th Five-Year Plan Period (2011BAD34B03-4), the Doctoral Program of Higher Education Research Fund (Instructor Dr. Class 20105103110001), and the National Natural Science Foundation of China (Grant No. 31272612).

References

Antoine, T.E., Mishra, Y.K., Trigilio, J., Tiwari, V., Adelung, R., Shukla, D., 2012. Prophylactic, therapeutic and neutralizing effects of zinc oxide tetrapod structures against herpes simplex virus type-2 infection. Antivir. Res. 96, 363–375.

Carlucci, M.J., Scolaro, L.A., Noseda, M.D., Cerezo, A.S., Damonte, E.B., 2004. Protective effect of a natural carrageenan on genital herpes simplex virus infection in mice. Antivir. Res. 64, 137–141.

Damonte, E.B., Matulewicz, M.C., Cerezo, A.S., 2004. Sulfated seaweed polysaccharides as antiviral agents. Curr. Med. Chem. 11, 2399–2419.

Dodgson, K., Price, R., 1962. A note on the determination of the ester sulphate content of sulphated polysacchrides. Biochem. J. 84, 106–110.

Gerber, P., Dugene, J.D., Adams, E.V., Sherman, J.H., 1958. Protective effect of seaweed extracts for chicken embryos infected with influenza B or Mumps virus. Proc. Soc. Exp. Biol. Med. 99, 590–593.

Guo, Y.F., Cheng, A.C., Wang, M.S., Zhou, Y., 2009a. Purification of anatid herpesvirus 1 particles by tangential-flow ultrafiltration and sucrose gradient ultracentrifugation. J. Virol. Methods 161, 1–6.

Guo, Z.H., Hu, Y.L., Wang, D.Y., Ma, X., Zhao, X.N., Zhao, B.K., Wang, J.M., Liu, P., 2009b. Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. Vaccine 27, 660–665.

Han, S.Q., Yoshida, D., Kanamoto, T., Nakashima, H., Uryu, T., Yoshida, T., 2010. Sulfated oligosaccharide cluster with polylysine core scaffold as a new anti-HIV dendrimer. Carbohydr. Polym. 80, 1111–1115.

Harden, A.E., Falshaw, R., Carnachan, M.S., Kern, R.E., Prichard, N.M., 2009. Virucidal activity of polysaccharide extracts from four algal species against herpes simplex virus. Antivir. Res. 83, 282–289.

Hayashi, T., Hayashi, K., Maeda, M., Kojima, I., 1996. Calcium spirulan, an inhibitor of enveloped virus replication, from a blue–green alga *Spirulina platensis*. J. Nat. Prod. 59, 83–87.

Heldwein, E.E., Krummenacher, C., 2008. Entry of herpesviruses into mammalian cells. Cell. Mol. Life Sci. 65, 1653–1668.

Huang, X.Y., Wang, D.Y., Hu, Y.L., Lu, Y., Guo, Z.H., Kong, X.F., Sun, J.L., 2008a. Effect of sulfated astragalus polysaccharide on cellular infectivity of infectious bursal disease virus. Int. J. Biol. Macromol. 42, 166–171.

Huang, X.Y., Hu, Y.L., Zhao, X.N., Lu, Y., Wang, J.M., Zhang, F., Sun, J.L., 2008b. Sulfated modification can enhance the adjuvant activity of astragalus polysaccharide for ND vaccine. Carbohydr. Polym. 73, 303–308.

Lee, E., Pavy, M., Young, N., Freeman, C., Lobigs, M., 2006. Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. Antivir. Res. 69, 31–38.

Lei, X.L., Zhang, M., 2012. Study on the impact of different processing methods on the polysaecharide content and peulustrin content in Chuanmingshen. Pharm. Clin. Chin. Materia Medica 3, 34–38.

Lian, B., Xu, C., Cheng, A., Wang, M., Zhu, D., Luo, Q., Jia, R., Bi, F., Chen, Z., Zhou, Y., Yang, Z., Chen, X., 2010. Identification and characterization of duck plague virus glycoprotein C gene and gene product. Virol. J. 7, 349.

Li, H., Shao, C., 1996. Study on anti-mutagenic effect of the Chuanmingshen polysaccharide. J. Yuzhou Univ. (Nat. Scien. Edit.) 4, 24–28.

Liu, J., Thorp, S.C., 2002. Cell surface heparan sulfate and its roles in assisting viral infections. Med. Res. Rev. 22, 1–25.

Liu, X.X., Wan, Z.J., Shi, L., Lu, X.X., 2011. Preparation and antiherpetic activities of chemically modified polysaccharides from *Polygonatum cyrtonema* Hua. Carbohydr. Polym. 83, 737–742.

Li, Y., Huang, B., Ma, X., Wu, J., Li, F., Ai, W., Song, M., Yang, H., 2009. Molecular characterization of the genome of duck enteritis virus. Virology 391, 151–161.

Lu, Y., Wang, D.Y., Hu, Y.L., Huang, X.Y., Wang, J.M., 2008. Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV. Carbohydr. Polym. 71, 180–186.

Ma, X., Guo, Z.H., Wang, D.Y., Hu, Y.L., Shen, Z.Q., 2010. Effects of sulfated polysaccharides and their prescriptions on immune response of ND vaccine in chicken. Carbohydr. Polym. 82, 9–13.

Mondal, B., Rasool, T.J., Ram, H., Mallanna, S., 2010. Propagation of vaccine strain of duck enteritis virus in a cell line of duck origin as an alternative production system to propagation in embryonated egg. Biologicals 38, 401–406.

Nyberg, K., Ekblad, M., Bergström, T., Freeman, C., Parish, C.R., Ferro, V., Trybala, E., 2004. The low molecular weight heparan sulfate-mimetic, PI-88, inhibits cell-to-cell spread of herpes simplex virus. Antivir. Res. 63, 15–24.

Pourianfar, H.R., Poh, C.L., Fecondo, J., Grollo, L., 2012. *In vitro* evaluation of the antiviral activity of heparan sulfate mimetic compounds against Enterovirus 71. Virus Res. 169, 22–29.

Pujol, A.C., Carlucci, J.M., Matulewicz, C.M., Damonte, B.E., 2007. Natural sulfated polysaccharides for the prevention and control of viral infections. Top. Hetero. Chem. 11, 259–281.

Qi, X.L., Yang, X.Y., Cheng, A.C., Wang, M.S., Guo, Y.F., Jia, R.Y., 2009. Replication kinetics of duck virus enteritis vaccine virus in ducklings immunized by the mucosal or systemic route using real-time quantitative PCR. Res. Vet. Sci. 1, 63–67.

Rabenstein, D.L., 2002. Heparin and heparan sulfate: structure and function. Nat. Prod. Rep. 19, 312–331.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E., 2008. Diseases of Poultry, twelfth ed. Blackwell Publishing, UK.

Siakallis, G., Spandidos, D.A., Sourvinos, G., 2009. Herpesviridae and novel inhibitors. Antivir. Ther. 14, 1051–1064.

Song, X., Xu, J., Yin, Z.Q., Jia, R.Y., Cheng, A.C., Deng, Y.X., Lü, C., Liang, X.X., Wang, Y., Yang, Z.X., Yao, X.P., Zhang, W., 2013. Effects of Polysaccharide from *Chuanminshen violaceum* on immune response of newcastle disease vaccine in chicken. Acta Sci. Vet. 41, 1104.

Spear, P.G., Longnecker, R., 2003. Herpesvirus entry: an update. J. Virol. 77, 10179–10185.

Talarico, L.B., Pujolm, C.A., Zibetti, R.G.M., Faria, P.C.S., Noseda, M.D., Duarte, M.E.R., Damontea, E.B., 2005. The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell. Antivir. Res. 66, 103–110.

Waarts, B.L., Aneke, O.J., Smit, J.M., Kimata, K., Bittman, R., Meijer, D.K., Wilschut, J., 2005. Antiviral activity of human lactoferrin: inhibition of alphavirus interaction with heparan sulfate. Virology 333, 284–292.

Wang, C.Y., Giambrone, J.J., Smith, B.F., 2002. Detection of duck hepatitis B virus DNA on filter paper by PCR and SYBR green dye-based quantitative PCR. J. Clin. Microbiol. 40, 2584–2590.

Wang, D.Y., Guo, Z.H., Ma, X., Hu, Y.L., Huang, X.Y., Fan, Y.P., Yang, S.J., Guo, L., 2010a. Effects of sulfated lentinan on cellular infectivity of avian infectious bronchitis virus. Carbohydr. Polym. 79, 461–465.

Wang, J.M., Hu, Y.L., Wang, D.Y., Liu, J., Zhang, J., Abula, S., Zhao, B., Ruan, S.L., 2010b. Sulfated modification can enhance the immune-enhancing activity of lycium barbarum polysaccharides. Cell. Immunol. 263, 219–223.

Wang, J.C., Höper, D., Beer, M., Osterrieder, N., 2011a. Complete genome sequence of virulent duck enteritis virus (DEV) strain 2085 and comparison with genome sequences of virulent and attenuated DEV strains. Virus Res. 160, 316–325.

Wang, W., Zhang, P., Hao, C., Zhang, X.E., Cui, Z.Q., Guan, H.S., 2011b. *In vitro* inhibitory effect of carrageenan oligosaccharide on influenza A H_1N_1 virus. Antivir. Res. 92, 237–246.

Wang, J.C., Osterrieder, N., 2011. Generation of an infectious clone of duck enteritis virus (DEV) and of a vectored DEV expressing hemagglutinin of H_5N_1 avian influenza virus. Virus Res. 159, 23–31.

Wijesekara, I., Pangestuti, R., Kim, S., 2011. Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydr. Polym. 84, 14–21.

Witvrouw, M., De Clercq, E., 1997. Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. Gen. Pharmacol. 29, 497–511.

Wu, Y., Cheng, A.C., Wang, M.S., Yang, Q., Zhu, D.K., Jia, R.Y., Chen, S., Zhou, Y., Wang, X.Y., Chen, X.Y., 2012a. Complete genomic sequence of Chinese virulent duck enteritis virus. J. Virol. 86, 5965.

Wu, Y., Cheng, A.C., Wang, M.S., Zhu, D.K., Jia, R.Y., Chen, S., Zhou, Y., Chen, X.Y., 2012b. Comparative genomic analysis of duck enteritis virus strains. J. Virol. 86, 13841–13842.

Xu, J., Song, X., Yin, Z.Q., Cheng, A.C., Jia, R.Y., Deng, Y.X., Ye, K.C., Shi, C.F., Lv, C., Zhang, W., 2012. Antiviral activity and mode of action of extracts from neem seed kernel against duck plague virus *in vitro*. Poult. Sci. 91, 2802–2807.

Yasuhara-Bell, J., Lu, Y.A., 2010. Marine compounds and their antiviral activities. Antiviral. Res. 86, 231–240.

Zhang, M., Yu, T., Su, X., Zhang, H., 2007. Physiochemical properties and the immunological activity of the Chuanmingshen polysaccharide. West China J. Pharm. Sci. 4, 396–398.

Zhao, X.N., Hu, Y.L., Wang, D.Y., Guo, L.W., Yang, S.J., Fan, Y.P., Zhao, B.K., Wang, Y.L., Abula, S., 2011. Optimization of sulfated modification conditions of tremella polysaccharide and effects of modifiers on cellular infectivity of NDV. Int. J. Biol. Macromol. 49, 44–49.