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In vitro antiviral activity of sulfated *Auricularia auricula* polysaccharides

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ABSTRACT

Total *Auricularia auricula* polysaccharide (AAP_t) was prepared by extracting and removing the proteins. Column chromatography was used to further graded it into AAP_1 and AAP_2. Three AAPs were modified by chlorosulfonic acid–pyridine method to obtain three sulfated AAPs (sAAPs), $sAAP_t$, $sAAP_1$ and $sAAP_2$, respectively. Three sAAPs and Newcastle disease virus (NDV) were added into cultivation system of chicken embryo fibroblast (CEF) in three manners, pre-, post- and simultaneous-adding polysaccharide with NDV respectively, taking three non-modified AAPs as control. Their anti-viral activities were compared by MTT method. The results showed that sAAPs and AAPs at a certain concentration could significantly inhibit the cellular infectivity of NDV in three manners. The effects of sAAPs were better than that of AAPs. It indicated that sulfated modification could enhance the antiviral activity of AAP. $sAAP_1$ and $sAAP_t$ possessed stronger activity and would be as the component of a new-type antiviral drug.

1. Introduction

Polysaccharide is the polymerizer of aldose or ketose by glycosidic bond. It widely exists in higher plants, fungi, algae, bacteria and cell membrane of animals and is one of the four basic substances constituting life (Leung, Liu, Koon & Fung, 2006). Polysaccharide is not only major structural component of organisms but also has complicated biological activities, such as enhancing immunity, anti-tumor, antivirus, anti-aging, lowering blood glucose, and stimulating haematogenesis (Yang & Zhang, 2009), and has little side effects (Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005). Sulfated polysaccharide is a class of polysaccharides with sulfation group in its hydroxyl and possesses many different or stronger biological activities in comparison with non-sulfated polysaccharide, such as anti-virus (Bandyopadhyay, Navid, Ghosh, Schnitzler, & Ray, 2011; Ghosh et al., 2009; Saha, Navid, Bandyopadhyay, Schnitzler, & Ray, 2012), antitumor (Chen et al., 2011; Wang & Zhang, 2009; Zhao et al., 2011), anti-coagulation (Alban & Franz, 2000), immune enhancement, hypoglycemic activity (Wang, Peng, et al., 2010) antioxidant capacity (Barahona, Encinas, Mansilla, Matsuhiro, & Zúñiga, 2012) and so on. Along with the increasing pursuit for strong or new biological activities, the molecular modification and structure improvement of polysaccharide has become an important research field.

Auricularia auricula is widely used as a medicine and food supplement in China, Korea and Viet Nam. *Auricularia auricula* polysaccharide (AAP) is the important active component of *Auricularia auricula* and has many actions, such as anti-tumor (Ma, Wang, Zhang, Zhang, & Ding, 2010; Misaki, Kakuta, Sasaki, Tanaka, & Miyaji, 1981), hypocholesterolemic activity (Cheung, 1996), hypolipidemia activity (Han & Xu, 2007), enhancing immunity, lowering blood glucose, anti-aging, anti-radiation anti-mutation and so on (Han & Xu, 2007; Song & Du, 2012; Zhang, 2001; Zhang, Ji, Qu & Wang, 2003) However there is no report to confirm whether it has the antiviral activity and sulfation modification can raise its antiviral activity.

In present research, the total *Auricularia auricula* polysaccharide (AAP_t) was prepared by extracting, removing the proteins and further grading it into AAP_1 and AAP_2 through column chromatography. Three sulfated AAPs (sAAPs), $sAAP_t$, $sAAP_1$ and $sAAP_2$,

Abbreviations: AAP, *Auricularia auricula* polysaccharide; sAAP, sulfated AAP; NDV, Newcastle disease virus; CEF, chicken embryo fibroblast; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; CSA, chlorosulfonic acid; DMSO, dimethyl sulfoxide; DS, degree of sulfation; DMF, dimethylformamide; MEM, Eagle's minimum essential medium; MM, maintenance medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Pyr, pyridine; SPF, specified-pathogens free.

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were prepared by chlorosulfonic acid–pyridine method (Li & Wang, 2008). Their effects of three sulfated sAAPs and three non-sulfated AAPs on cellular infectivity of Newcastle disease virus (NDV) were compared. The purpose of this research was to probe into the probability of improving the antiviral activity of APP through sulfation modification, screen out the best sAAP and offer theory evidence for developing antiviral drug.

2. Material and methods

2.1. Auricularia auricular polysaccharide

The black fungus was produced in northeast of China and purchased from a Carrefour supermarket in Nanjing city, Jiangsu province, PR China. The crude total AAP was extracted by water decoction and ethanol precipitation (Wang, Guo, et al., 2010) and purified by Sevages' method to eliminate protein to obtain total AAP (AAP_t) (Huang et al., 2008), and permeated through DEAE Sephadex A-25 cellulose column chromatography (Zhang, Wang, Zhang & Wang, 2011). After freeze drying, two purified AAPs were obtained and coded as AAP_1 and AAP_2. The polysaccharide contents of AAP_t, AAP_1 and AAP_2 determined by phenol–sulfuric acid method (Li & Wang, 2008; Yu, Yang, Liu & Liu, 2009) were 60.92%, 25.97% and 12.57%, respectively.

2.2. Reagents

Chlorosulfonic acid (CSA) was purchased from of Shanghai Experimental Reagent Ltd.; Pyridine (Pyr) and dimethylformamide (DMF) were purchased from Chemical Reagent Ltd. of National Drug Group; Dimethyl sulfoxide (DMSO, No. 090601) was produced by Kemiou Institute of Chemical Engineering in Tianjin. All other chemicals used were analytical grade.

Eagle's minimum essential medium (MEM, Gibco Co.) supplemented with 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 5% fetal bovine serum, was used as nutritive medium. For maintenance medium (MM), the serum concentration was reduced to 2%. Trypsin (Amresco) was dissolved into 0.25% with CMF-PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co.) was dissolved into 5 mg mL^{-1} with CMF-PBS. These reagents were filtered through a 0.22 μm millipore membrane filter. MEM and MM were stored at 4 °C, trypsin solution, −20 °C, MTT solution was kept at 4 °C in a dark bottle and finished within a month.

2.3. Sulfation modification of AAP

Three sAAPs, $sAAP_t$, $sAAP_1$ and $sAAP_2$, were prepared by the chlorosulfonic acid–pyridine method with the same modified conditions as the reference (Li, 2006; Li & Wang, 2008): the ratio of chlorosulfonic acid to pyridine of 5:1, reaction temperature of 50 °C and reaction time of 1.5 h. Their polysaccharide contents were 23.74%, 12.60% and 10.14%, respectively.

2.4. Determination and identification of DS

The sulphur contents of three sAAPs were determined by Antonopoulos' method (Dodgson & Price, 1962; Zhang, Wang, Yang, et al., 2011). A calibration curve was constructed with sodium sulfate as standard. The degree of sulfation (DS) was calculated according to the equation: $DS = (1.62 \times S\%)/(32 - 1.02 \times S\%)$. The DS of $sAAP_t$, $sAAP_1$ and $sAAP_2$ were 0.22, 1.46, and 1.19, respectively.

FT-IR spectra of the AAP_t and $sAAP_t$ were recorded by Nicolet FT-IR 200 spectrophotometer (Nicolet) and KBr pellets method (Yang & Zhang, 2009; Zhang, Wang, Yang, et al., 2011). In the spectra of AAP_t and $sAAP_t$, the band in the region of 3600–3200 cm^{-1} corresponds to the hydroxyl stretching vibration and in the region of 3000–2800 cm^{-1} the band corresponds to a weak C—H stretching vibration. The bands attributed to C—O—C stretching vibrations appeared at about 1400–1000 cm^{-1}. This amalgamation indicates that the AAP_t and $sAAP_t$ were polysaccharides. In comparison with the spectrogram of AAP_t, the FT-IR spectroscopy of $sAAP_t$ showed two characteristic absorption bands, one at 1235 cm^{-1} describing an asymmetrical S=O stretching vibration and the other at 819 cm^{-1} representing a symmetrical C—O—S vibration associated with a C—O—SO_3 group, which signified that $sAAP_t$ was successfully sulfated.

2.5. Cells and viruses

CEF were prepared with 10 days old chick embryo (purchased from Nanjing Pharmaceuticals and Instruments Factory). The cells was diluted into 1×10^6 mL^{-1} with MEM and inoculated into 96 well culture plates at 38.5 °C for 24 h in a humid atmosphere of 5% CO_2 for used (Wang, Hu, et al., 2010).

NDV (La Sota strain) was bought from Nanjing Pharmaccutical and Instruments Factory and proliferated by inoculating SPF chicken embryo. $TCID_{50}$ of the virus liquid was 1×10^{-5} by Reed–Mueeh assay (Huang et al., 2008; Yin & Liu, 1997). It was diluted into 1×10^{-3} (100 $TCID_{50}$) with MM for the test.

2.6. Cytotoxicity analysis

Three sulfated $sAAP_t$, $sAAP_1$ and $sAAP_2$ and three non sulfated AAP_t, AAP_1 and $sAAP_2$ were dissolved with MM into 11 concentrations from 1000 to 0.98 μg mL^{-1}, for the test. CEF monolayers in 96-well plates were exposed to polysaccharides at series of concentrations, four wells each concentration. After a culturing for 72 h at 38.5 °C in a humid atmosphere of 5% CO_2, the cytopathic effect (CPE) was observed and CEF livingness was tested by MTT method (Ciapetti, Cenni, Pratelli & Pizzoferrato, 1993; Zhao et al., 2011). Twenty microliters of MTT was added to each well, incubated at 38.5 °C for 4 h in a humid atmosphere of 5% CO_2, the supernatant was removed and 100 μL of DMSO was added. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance at 570 nm (A_{570} value) each well was measured by microliter enzyme-linked immunosorbent assay reader (Fan et al., 2011). When A_{570} values of polysaccharide group were not significantly lower than that of the cell control group, it indicated that the polysaccharides had no cytotoxicity, and the corresponding concentrations were considered as maximal safety concentration for CEF (Liu, Hu, Zhang & Kong, 2002).

2.7. Antiviral assays

Three sAAPs and three AAPs were dissolved with MM into five concentrations from 3.91 to 0.244 μg mL^{-1} based on above-mentioned cytotoxicity analysis. When CEF grew into monolayer, the serial two-fold dilutions of polysaccharides and NDV were added respectively in three sample-adding modes as follow (Fan et al., 2011).

Pre-adding polysaccharide: sAAPs solutions were added into CEF plate firstly, 100 μL per well, four wells per concentration. After incubation for 2 h, the polysaccharide was removed, the cells were washed twice with Hanks' solution, and 100 μL of the virus solution was added into per well.

Post-adding polysaccharide: The virus solution was added into CEF plate first. After incubation for 2 h, the virus solution was removed and the cells were washed twice with Hanks' solution. sAAPs solutions were added, four wells each concentration.

Table 1
The cytotoxicity of sAPPs to CEF (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sAAP_t$	$sAAP_1$	$sAAP_2$	AAP_t	AAP_1	AAP_2
1000	0.160 ± 0.018^{e}	0.284 ± 0.025^{e}	0.190 ± 0.017^{e}	0.226 ± 0.005^{e}	0.182 ± 0.017^{d}	0.111 ± 0.010^{d}
500	0.171 ± 0.028^{e}	0.278 ± 0.023^{e}	0.187 ± 0.015^{e}	0.316 ± 0.013^{d}	0.194 ± 0.014^{cd}	0.120 ± 0.017^{d}
250	0.164 ± 0.019^{e}	0.291 ± 0.020^{e}	0.244 ± 0.029^{d}	0.360 ± 0.007^{bc}	0.197 ± 0.011^{bcd}	0.150 ± 0.017^{d}
125	0.162 ± 0.017^{e}	0.363 ± 0.035^{d}	0.376 ± 0.028^{c}	0.368 ± 0.012^{bc}	0.236 ± 0.013^{bcd}	0.160 ± 0.016^{d}
62.5	0.283 ± 0.016^{d}	0.357 ± 0.021^{d}	0.401 ± 0.020^{bc}	0.414 ± 0.017^{a}	0.247 ± 0.020^{bc}	0.260 ± 0.013^{c}
31.25	0.425 ± 0.015^{c}	0.414 ± 0.020^{c}	0.425 ± 0.019^{b}	0.436 ± 0.021^{a}	0.254 ± 0.014^{b}	0.283 ± 0.032^{c}
15.63	0.416 ± 0.019^{c}	0.427 ± 0.014^{c}	0.428 ± 0.019^{b}	0.433 ± 0.010^{a}	0.403 ± 0.018^{a}	0.407 ± 0.011^{b}
7.81	0.417 ± 0.015^{c}	0.475 ± 0.019^{b}	0.471 ± 0.021^{a}	0.339 ± 0.009^{cd}	0.445 ± 0.018^{a}	0.389 ± 0.012^{b}
3.91	0.503 ± 0.026^{b}	0.495 ± 0.025^{b}	0.474 ± 0.017^{a}	0.349 ± 0.010^{cb}	0.412 ± 0.013^{a}	0.497 ± 0.019^{a}
1.95	0.554 ± 0.025^{a}	0.555 ± 0.025^{a}	0.482 ± 0.018^{a}	0.375 ± 0.009^{b}	0.413 ± 0.015^{a}	0.505 ± 0.017^{a}
0. 98	0.556 ± 0.029^{a}	0.549 ± 0.019^{a}	0.493 ± 0.028^{a}	0.374 ± 0.010^{b}	0.414 ± 0.022^{a}	0.518 ± 0.022^{a}
Cell	0.535 ± 0.011^{ab}	0.505 ± 0.025^{b}	0.505 ± 0.025^{a}	0.380 ± 0.010^{b}	0.435 ± 0.036^{a}	0.535 ± 0.011^{a}

Column data marked without the same superscripts (a–e) differ significantly ($P < 0.05$).

Table 2
The cellular A_{570} values of every group in pre-adding polysaccharide.

Concentration ($\mu g\,mL^{-1}$)	$sAAP_t$	$sAAP_1$	$sAAP_2$	AAP_t	AAP_1	AAP_2
3.906	0.487 ± 0.035^{b}	0.488 ± 0.030^{abc}	0.493 ± 0.062^{b}	0.497 ± 0.032^{ab}	0.397 ± 0.035^{b}	0.208 ± 0.023^{d}
1.953	0.452 ± 0.027^{bc}	0.466 ± 0.037^{bcd}	0.406 ± 0.051^{bcd}	0.349 ± 0.061^{c}	0.360 ± 0.018^{bc}	0.232 ± 0.010^{d}
0.977	0.497 ± 0.030^{b}	0.521 ± 0.059^{ab}	0.393 ± 0.063^{bcd}	0.412 ± 0.059^{bc}	0.320 ± 0.036^{cd}	0.333 ± 0.026^{b}
0.488	0.313 ± 0.030^{d}	0.355 ± 0.086^{cd}	0.424 ± 0.003^{bc}	0.352 ± 0.070^{c}	0.280 ± 0.037^{d}	0.280 ± 0.011^{c}
0.244	0.434 ± 0.084^{bc}	0.468 ± 0.058^{bcd}	0.311 ± 0.021^{d}	0.304 ± 0.061^{c}	0.255 ± 0.012^{d}	0.212 ± 0.012^{d}
Virus control	0.334 ± 0.029^{cd}	0.334 ± 0.029^{dc}	0.334 ± 0.029^{cd}	0.334 ± 0.029^{c}	0.138 ± 0.008e	0.138 ± 0.008^{e}
Cell control	0.623 ± 0.022^{a}	0.623 ± 0.022^{a}	0.623 ± 0.022^{a}	0.623 ± 0.022^{a}	0.641 ± 0.008^{a}	0.641 ± 0.008^{a}

Column data marked without the same superscripts (a–e) differ significantly ($P < 0.05$).

Simultaneous-adding polysaccharide and virus after mixed: sAAPs at five concentrations were mixed respectively with virus solutions and incubated at 4 °C for 2 h, then added into CEF plate, four wells per concentration. At the same time, the NDV control group (only adding virus), cell control group (only adding MM) and blank group (no cell) were designed. All the plates were placed into 5% CO_2 incubator at 38.5 °C. When the NDV control group appeared obviously cytopathic effect (72 h), the CEF livingness (A_{570} value) was measured by the MTT method (Verma et al., 2010). The virus inhibitory rate was calculated based on the formula (Fan et al., 2011): Virus inhibitory rate = $(\bar{A}_{\text{polysaccharide+virus}} - \bar{A}_{\text{virus control}})/(\bar{A}_{\text{cell control}} - \bar{A}_{\text{virus control}}) \times 100\%$. The A_{570} values and virus inhibitory rate were considered as the indicator of antiviral activity.

2.8. Statistical analysis

Data are expressed as the mean ± S.D. Duncan and LSD's multiple range test was used to determine the difference among AAP and control groups with the software SPSS 16.0. Significant differences were considered as $P < 0.05$.

3. Results

3.1. Cytotoxicity

The A_{570} values of every polysaccharide group are listed in Table 1. The A_{570} values of $sAAP_t$ and AAP_2 at 3.91–0.98 $\mu g\,mL^{-1}$, $sAAP_1$ and $sAAP_2$ at 7.81–0.98 $\mu g\,mL^{-1}$, AAP_t at 250–125 $\mu g\,mL^{-1}$, AAP_1 at 15.63–0.98 $\mu g\,mL^{-1}$ group were not significantly lower than that of corresponding cell control group ($P > 0.05$). Therefore, $sAAP_t$ and AAP_2 at 3.91 $\mu g\,mL^{-1}$, $sAAP_1$ and $sAAP_2$ at 7.81 $\mu g\,mL^{-1}$, AAP_t at 250 $\mu g\,mL^{-1}$, AAP_1 at 15.63 $\mu g\,mL^{-1}$. These concentrations could be considered as their maximal safety concentrations. In order to make the comparison at the same level, their maximal safe concentrations were supposed to be as 3.91 $\mu g\,mL^{-1}$.

3.2. Antiviral activities in pre-adding polysaccharide

The A_{570} values of every group are listed in Table 2. The A_{570} values of $sAAP_t$ and $sAAP_1$ at 3.906 and 0.977 $\mu g\,mL^{-1}$, $sAAP_2$ at 3.906 $\mu g\,mL^{-1}$, AAP_t at 3.906 $\mu g\,mL^{-1}$, AAP_1 and AAP_2 at 3.906–0.244 $\mu g\,mL^{-1}$ groups were significantly higher than that of corresponding virus control group ($P < 0.05$).

The virus inhibitory rates of every group are listed in Fig. 1. The virus inhibitory rate in $sAAP_1$ group (43.38%) was the highest, and the following was the AAP_1 (36.71%), $sAAP_t$ (35.40%), $sAAP_2$ (24.60%), AAP_2 (22.90%) and AAP_t (16.78%).

3.3. Antiviral activities in post-adding polysaccharide

The A_{570} values of every group are listed in Table 3. The A_{570} values of $sAAP_t$ at 0.488 $\mu g\,mL^{-1}$, $sAAP_1$ at 3.906–0.244 $\mu g\,mL^{-1}$, $sAAP_2$ at 3.906–0.488 $\mu g\,mL^{-1}$, AAP_t at 1.953 $\mu g\,mL^{-1}$, AAP_1 at 1.953–0.977 $\mu g\,mL^{-1}$ groups were significantly higher than that of corresponding virus control ($P < 0.05$).

The virus inhibitory rates of every group are listed in Fig. 2. The virus inhibitory rate of $sAAP_1$ group was the highest and the following was $sAAP_t$ group. This two groups were significantly higher than that of AAP_2 group ($P < 0.05$). The next were AAP_1 (51.37%), AAP_t (42.66%), $sAAP_2$ (36.64%)and AAP_2 (33.67%).

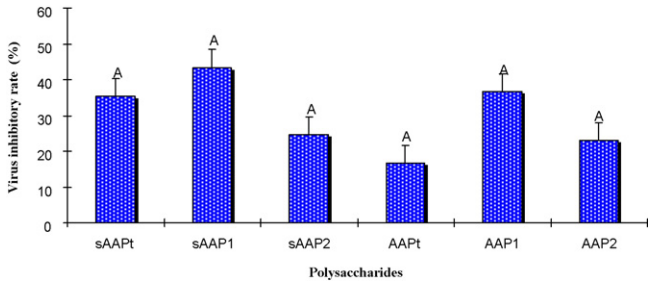

Fig. 1. The virus inhibitory rate of every group in pre-adding polysaccharide. Bars marked the same letter (A) differ not significantly ($P > 0.05$).

Table 3
The cellular A_{570} values of every group in post-adding polysaccharide.

Concentration ($\mu g\,mL^{-1}$)	$sAAP_t$	$sAAP_1$	$sAAP_2$	AAP_t	AAP_1	AAP_2
3.906	0.363 ± 0.035^{bc}	0.398 ± 0.035^{b}	0.160 ± 0.023^{de}	0.285 ± 0.032^{b}	0.325 ± 0.010^{bc}	0.335 ± 0.026^{b}
1.953	0.395 ± 0.011^{bc}	0.360 ± 0.017^{bc}	0.183 ± 0.010^{d}	0.488 ± 0.023^{a}	0.405 ± 0.018^{b}	0.313 ± 0.022^{bc}
0.977	0.311 ± 0.041^{bc}	0.323 ± 0.037^{cd}	0.283 ± 0.026^{b}	0.343 ± 0.014^{b}	0.408 ± 0.019^{b}	0.305 ± 0.053^{bc}
0.488	0.413 ± 0.034^{b}	0.280 ± 0.038^{d}	0.230 ± 0.011^{c}	0.313 ± 0.013^{b}	0.270 ± 0.018^{c}	0.300 ± 0.018^{bc}
0.244	0.388 ± 0.076^{bc}	0.253 ± 0.011^{d}	0.162 ± 0.012^{de}	0.265 ± 0.026^{b}	0.328 ± 0.011^{bc}	0.211 ± 0.023^{c}
Virus control	0.272 ± 0.041^{c}	0.137 ± 0.010^{e}	0.137 ± 0.010^{e}	0.272 ± 0.041^{b}	0.272 ± 0.041^{c}	0.272 ± 0.041^{bc}
Cell control	0.568 ± 0.017^{a}	0.643 ± 0.008^{a}	0.643 ± 0.008^{a}	0.568 ± 0.017^{a}	0.568 ± 0.017^{a}	0.568 ± 0.017^{a}

Column data marked without the same superscripts (a–e) differ significantly ($P < 0.05$).

Table 4
The cellular A_{570} values of every group in simultaneous-adding polysaccharide and virus.

Concentration ($\mu g\,mL^{-1}$)	$sAAP_t$	$sAAP_1$	$sAAP_2$	AAP_t	AAP_1	AAP_2
3.906	0.444 ± 0.039^{bc}	0.438 ± 0.023^{ab}	0.416 ± 0.011^{b}	0.415 ± 0.059^{bc}	0.394 ± 0.020^{b}	0.301 ± 0.018^{bc}
1.953	0.428 ± 0.041^{bc}	0.442 ± 0.026^{ab}	0.418 ± 0.033^{b}	0.443 ± 0.034^{b}	0.385 ± 0.033^{bc}	0.312 ± 0.010^{bc}
0.977	0.515 ± 0.028^{ab}	0.417 ± 0.025^{b}	0.408 ± 0.021^{b}	0.282 ± 0.017^{d}	0.268 ± 0.017^{d}	0.322 ± 0.023^{b}
0.488	0.393 ± 0.055^{c}	0.454 ± 0.008^{ab}	0.356 ± 0.019^{b}	0.302 ± 0.031^{d}	0.301 ± 0.052^{cd}	0.343 ± 0.036^{b}
0.244	0.454 ± 0.023^{abc}	0.454 ± 0.032^{ab}	0.352 ± 0.014^{b}	0.337 ± 0.026^{cd}	0.402 ± 0.012^{b}	0.303 ± 0.029^{bc}
Virus control	0.238 ± 0.029^{d}	0.250 ± 0.022^{c}	0.250 ± 0.022^{c}	0.238 ± 0.029^{d}	0.238 ± 0.029^{d}	0.238 ± 0.029^{c}
Cell control	0.556 ± 0.021^{a}	0.519 ± 0.032^{a}	0.519 ± 0.032^{a}	0.556 ± 0.021^{a}	0.556 ± 0.021^{a}	0.556 ± 0.021^{a}

Column data marked without the same superscripts (a–d) differ significantly ($P < 0.05$).

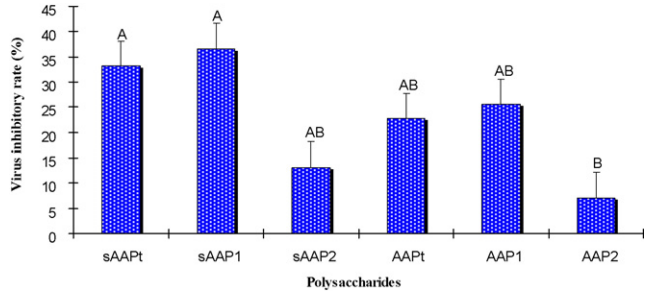

Fig. 2. The virus inhibitory rate in post-adding polysaccharide. Bars marked without the same letters (A–B) differ significantly ($P < 0.05$).

3.4. Antiviral activities in simultaneous-adding polysaccharide and virus

The A_{570} values of every group are listed in Table 4. The A_{570} values of $sAAP_t$, $sAAP_1$ and $sAAP_2$ at 3.906–0.244 $\mu g\,mL^{-1}$, AAP_t at 3.906–1.593 $\mu g\,mL^{-1}$, AAP_1 at 3.906–1.593 $\mu g\,mL^{-1}$ and 0.244 $\mu g\,mL^{-1}$, AAP_2 at 0.977–0.488 $\mu g\,mL^{-1}$groups were significantly higher than those of corresponding virus control ($P < 0.05$).

The virus inhibitory rates of every group are listed in Fig. 3. The virus inhibitory rate in $sAAP_1$ (70.90%) and $sAAP_t$ (65.75%) group was the highest, significantly higher than that in APPs groups ($P < 0.05$), following was $sAAP_2$ (51.95%) were significantly higher than that of AAP_2 (24.74%) group ($P < 0.05$).

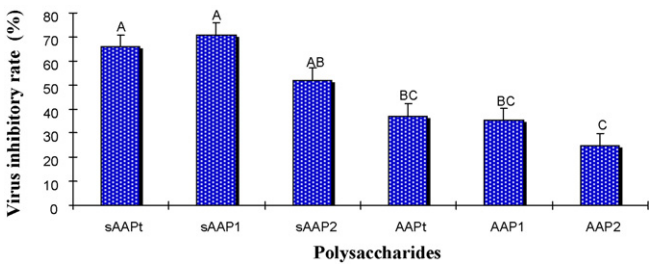

Fig. 3. The virus inhibitory rate in simultaneous adding polysaccharides and virus. Bars marked without the same letters (A–C) differ significantly ($P < 0.05$).

4. Discussion

The A_{570} value is an index to reflect living cells (Ma, Guo, Wang, Hu, & Shen, 2010). The higher A_{570} value becomes, the more living cells is and the better antiviral activity polysaccharide has. When the A_{570} values of polysaccharide were not significantly lower than those of their cell control group, it indicated that the polysaccharide had no cytotoxicity, and the corresponding concentration could be considered as the maximal safety concentration for CEF (Liu et al., 2002). The experimental results showed that between the A_{570} values of $sAAP_t$ and AAP_2 at 3.91–0.98 $\mu g\,mL^{-1}$, $sAAP_1$ and $sAAP_2$ at 7.81–0.98 $\mu g\,mL^{-1}$, AAP_t at 250–125 $\mu g\,mL^{-1}$, AAP_1 at 15.63–0.98 $\mu g\,mL^{-1}$ and the cell control group, there were not significant difference, and this indicated that sAAPs and AAPs could promote the cell growth within the certain concentration ranges.

Three sample-adding modes in this study are respectively corresponding to preservation, treatment and sterilization of three clinical administrations. The A_{570} value reflects not only the quantity of living cells but also the effect of polysaccharide to inhibit virus infection (Kong et al., 2004). The experimental results showed that in pre-adding modes, the A_{570} values in $sAAP_t$ and $sAAP_1$ at 2 concentrations and $sAAP_2$ at 1 concentration group were significantly higher than that of corresponding virus control group, which indicated that they could prevent NDV infection. During post-adding polysaccharide, the A_{570} values in $sAAP_t$ at 1 concentration, $sAAP_1$ at 5 concentrations and $sAAP_2$ at 3 concentration groups were significantly higher than that of corresponding virus control, which indicated that they could treat NDV infection. During simultaneous-adding polysaccharides and virus after mixed, the A_{570} values of sAAPs groups at all concentration were significantly higher than that of corresponding virus control group, which indicated that they could directly kill NDV. These results confirmed that three sAAPs at the suitable dose had a significant antiviral effect, sulfation modification could enhance the antiviral activity of AAP.

The virus inhibitory rate directly reflects the antiviral potency. In three drug-adding modes, the virus inhibitory rates of three sAAPs were significantly or numerically higher than that of corresponding non-modified AAPs, which confirmed again that sulfation modification could enhance antiviral action of APP. Many researches also confirmed that sulfation modification of polysaccharides extracted from Chinese herbal medicine had antiviral activity on some

viruses in vitro (Ghosh et al., 2009; Huang et al., 2008; Ma, Guo, et al., 2010; Zhang et al., 2011). To make a comparison of three sAAPs, it could be seen that $sAAP_1$ in three drug-adding modes, $sAAP_t$ in post- and simultaneous-adding modes were presented the highest virus inhibitory rate. This showed that the antiviral activities of all sulfated sAAPs were stronger than that of non-sulfated AAPs, which confirmed again that sulfation modification could enhance antiviral activity of AAP. Therefore, $sAAP_1$ and $sAAP_t$ would be as the component of a antiviral prescription.

As for antiviral mechanism of sulfated polysaccharide, it is considered that SO_4^{2-} polyanions of sulfated polysaccharide can combine with virus or cells, thus prohibit virus adsorption or inhibit some step of virus replication after entering the cell (Bagasra, Whittle, Heins & Pomerantz, 1991). Some scholars found that sulfated polysaccharide could combine with positive ion of V_3 area in envelope protein gp-120 of HIV-1 to cover the molecule of gp-20 so that the virus could not combine with CD_4 receptor of susceptibility cells (Artan, Karadeniz, Karagozlu, Kim, & Kim, 2010).

In conclusion, sulfated modification could enhance the antivirus activity of AAP. $sAAP_1$ and $sAAP_t$ possessed stronger activity and would be recommended as the component of a new antiviral drug.

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References

Alban, S., & Franz, G. (2000). Characterization of the anticoagulant actions of a semisynthetic curdlan sulfate. *Thrombosis Research*, *99*(4), 377–388.

Artan, M., Karadeniz, F., Karagozlu, M., Kim, M., & Kim, S. (2010). Anti-HIV-1 activity of low molecular weight sulfated chitooligosaccharides. *Carbohydrate Research*, *345*(5), 656–662.

Bagasra, O., Whittle, P., Heins, B., & Pomerantz, R. (1991). Anti-human immunodeficiency virus type 1 activity of sulfated monosaccharides: Comparison with sulfated polysaccharides and other polyions. *Journal of Infectious Diseases*, *164*(6), 1082–1090.

Bandyopadhyay, S., Navid, M., Ghosh, T., Schnitzler, P., & Ray, B. (2011). Structural features and in vitro antiviral activities of sulfated polysaccharides from Sphacelaria indica. *Phytochemistry*, *72*(2–3), 276–283.

Barahona, T., Encinas, M., Mansilla, A., Matsuhiro, B., & Zúñiga, E. (2012). A sulfated galactan with antioxidant capacity from the green variant of tetrasporic Gigartina skottsbergii (Gigartinales, Rhodophyta). *Carbohydrate Research*, *347*(1), 114–120.

Chen, T., Li, B., Li, Y., Zhao, C., Shen, J., & Zhang, H. (2011). Catalytic synthesis and antitumor activities of sulfated polysaccharide from Gynostemma pentaphyllum Makino. *Carbohydrate Polymers*, *83*(2), 554–560.

Cheung, P. (1996). The hypocholesterolemic effect of two edible mushrooms: *Auricularia auricula* (tree-ear) and Tremella fuciformis (white jelly-leaf) in hypercholesterolemic rats1. *Nutrition Research*, *16*(10), 1721–1725.

Ciapetti, G., Cenni, E., Pratelli, L., & Pizzoferrato, A. (1993). In vitro evaluation of cell/biomaterial interaction by MTT assay. *Biomaterials*, *14*(5), 359–364.

Dodgson, K., & Price, R. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*, 106–110.

Fan, Y., Liu, J., Wang, D., Hu, Y., Yang, S., Wang, J., et al. (2011). Epimedium polysaccharide and propolis flavone can synergistically inhibit the cellular infectivity of NDV and improve the curative effect of ND in chicken. *International Journal of Biological Macromolecules*, *48*(3), 439–444.

Ghosh, T., Chattopadhyay, K., Marschall, M., Karmakar, P., Mandal, P., & Ray, B. (2009). Focus on antivirally active sulfated polysaccharides: From structure–activity analysis to clinical evaluation. *Glycobiology*, *19*(1), 2–15.

Han, C., & Xu, L. (2007). Studies on the extraction, purification and hypolipidemia activity of auricularia auricular polysaccharides. *Journal of Chinese Institute of Food Science and Technology*, *7*(1), 53–57.

Huang, X., Wang, D., Hu, Y., Lu, Y., Guo, Z., Kong, X., et al. (2008). Effect of sulfated astragalus polysaccharide on cellular infectivity of infectious bursal disease virus. *International Journal of Biological Macromolecules*, *42*(2), 166–171.

Kong, X., Hu, Y., Wang, D., Li, X., Liu, J., Zhang, B., et al. (2004). Effects of Chinese herbal medicinal ingredients mixed with NDV on viral infectivity. *Journal of Nanjing Agricultural University*, *27*(2), 90–93.

Leung, M., Liu, C., Koon, J., & Fung, K. (2006). Polysaccharide biological response modifiers. *Immunology Letters*, *105*(2), 101–114.

Li, G. (2006). Application, function, and molecular modification of *Auricularia auricula* polysaccharide. Doctor of Agriculture, Ph.D. thesis (pp. 1, 66). Jilin: Northeast Forestry University.

Li, G., & Wang, Z. (2008). Sulfated esterifying technology of polysaccharide from *Auricularia auricula* and IR spectrum analysis. *Journal of Northeast Forestry University*, *36*(12), 66–68.

Liu, J., Hu, Y., Zhang, B., & Kong, X. (2002). The safety concentration of several components in natural drugs on CEF. *Progress in Veterinary Medicine*, *23*, 88–91.

Ma, X., Guo, Z., Wang, D., Hu, Y., & Shen, Z. (2010). Effects of sulfated polysaccharides and their prescriptions on immune response of ND vaccine in chicken. *Carbohydrate Polymers*, *82*(1), 9–13.

Ma, Z., Wang, J., Zhang, L., Zhang, Y., & Ding, K. (2010). Evaluation of water soluble [beta]-d-glucan from Auricularia auricular-judae as potential anti-tumor agent. *Carbohydrate Polymers*, *80*(3), 977–983.

Misaki, A., Kakuta, M., Sasaki, T., Tanaka, M., & Miyaji, H. (1981). Studies on interrelation of structure and antitumor effects of polysaccharides: Antitumor action of periodate-modified, branched (1 → 3)-[beta]-glucan of *auricularia auricula*-judae, and other polysaccharides containing (1 → 3)-glycosidic linkages. *Carbohydrate Research*, *92*(1), 115–129.

Saha, S., Navid, M., Bandyopadhyay, S., Schnitzler, P., & Ray, B. (2012). Sulfated polysaccharides from Laminaria angustata: Structural features and in vitro antiviral activities. *Carbohydrate Polymers*, *87*(1), 123–130.

Schepetkin, I., Faulkner, C., Nelson-Overton, L., Wiley, J., & Quinn, M. (2005). Macrophage immunomodulatory activity of polysaccharides isolated from Juniperus scopolorum. *International Immunopharmacology*, *5*(13-14), 1783–1799.

Song, G., & Du, Q. (2012). Structure characterization and antitumor activity of an α,β-glucan polysaccharide from Auricularia polytricha. *Food Research International*, *45*(1), 381–387.

Verma, A., Prasad, K., Singh, A., Nyati, K., Gupta, R., & Paliwal, V. (2010). Evaluation of the MTT lymphocyte proliferation assay for the diagnosis of neurocysticercosis. *Journal of Microbiological Methods*, *81*(2), 175–178.

Wang, D., Guo, Z., Ma, X., Hu, Y., Huang, X., Fan, Y., et al. (2010). Effects of sulfated lentinan on cellular infectivity of avian infectious bronchitis virus. *Carbohydrate Polymers*, *79*(2), 461–465.

Wang, J., Hu, Y., Wang, D., Zhang, F., Zhao, X., Abula, S., et al. (2010). Lycium barbarum polysaccharide inhibits the infectivity of Newcastle disease virus to chicken embryo fibroblast. *International Journal of Biological Macromolecules*, *46*(2), 212–216.

Wang, Y., Peng, Y., Wei, X., Yang, Z., Xiao, J., & Jin, Z. (2010). Sulfation of tea polysaccharides: Synthesis, characterization and hypoglycemic activity. *International Journal of Biological Macromolecules*, *46*(2), 270–274.

Wang, X., & Zhang, L. (2009). Physicochemical properties and antitumor activities for sulfated derivatives of lentinan. *Carbohydrate Research*, *344*(16), 2209–2216.

Yang, L., & Zhang, L. (2009). Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. *Carbohydrate Polymers*, *76*(3), 349–361.

Yu, W., Yang, X., Liu, W., & Liu, F. (2009). Assay study on content of polysaccharides in ficus carica by phenol–sulfuric acid method. *Food Science and Technology*, *10*(34), 256-258, 262.

Yin, Z., & Liu, J. H. (Eds.). (1997). *Animal virology*. Beijing: Science Press.

Zhang, C. (2001). Progress of pharmaceutical studies on *Auricularia auricula*. *Chinese Journal of Traditional Medical Science and Technology*, *5*, 339–340.

Zhang, X., Ji, Y., Qu, Z., & Wang, J. (2003). Proceeding of pharmacological research on *Auricularia auricula* polysaccharide. *Chinese Journal of Microecology*, *15*(6), 373–374.

Zhang, H., Wang, Z.-Y., Yang, L., Yang, X., Wang, X., & Zhang, Z. (2011). In vitro antioxidant activities of sulfated derivatives of polysaccharides extracted from Auricularia auricular. *International Journal of Molecular Sciences*, *12*(5), 3288–3302.

Zhang, H., Wang, Z., Zhang, Z., & Wang, X. (2011). Purified Auricularia auricular-judae polysaccharide (AAP I-a) prevents oxidative stress in an ageing mouse model. *Carbohydrate Polymers*, *84*(1), 638–648.

Zhao, X., Hu, Y., Wang, D., Guo, L., Yang, S., Fan, Y., et al. (2011). Optimization of sulfated modification conditions of tremella polysaccharide and effects of modifiers on cellular infectivity of NDV. *International Journal of Biological Macromolecules*, *49*(1), 44–49.