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Effects of sulfated lentinan on cellular infectivity of avian infectious bronchitis virus

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ABSTRACT

In order to investigate the possibility of sulfating modification in improving the anti-viral activity of lentinan (LNT), three kinds of sulfated LNTs (sLNT), $sLNT_1$, $sLNT_2$ and $sLNT_3$ with the degree of substitution of 0.69, 0.98 and 1.37 were prepared by chlorosulfonic acid–pyridine method with three modification condition, respectively. The sLNTs and avain infectious bronchitis virus (IBV) were added into chicken embryo fibroblast (CEF) in three manners, pre-adding polysaccharide, post-adding polysaccharide and mixed adding polysaccharide with IBV, respectively, taking non-modified LNT as control. The anti-viral activities of three sLNTs were compared by MTT method. The results showed that all of sLNTs and LNT at a certain concentration could significantly inhibit IBV growth in three manners. The effects of modified sLNTs were better than that of non-modified LNT. It indicated that sulfated modification could enhance the anti-viral activity of LNT.

1. Introduction

In recent years, polysaccharides, including natural polysaccharide and synthesized polysaccharide, are studied widely due to their various bioactivities (Bendjeddou, Lalaoui, & Satta, 2003; Mahmoud, Vladimir, Jacov, & Shoshana, 2002; Masahiko, Masahiro, Koichi, & Shiro, 1995). However, some polysaccharides appear lower bioactivity. Along with the increasing pursuit for manifold biological activities, molecular modification and structure reconstruction of polysaccharide become an important research field (Liu & Sun, 2005). The sulfated modification is one of the commonly used methods.

Sulfated polysaccharide is a kind of polysaccharide with sulfated group in its hydroxyls. It has different or stronger biological activities, such as anti-viral (Lu, Wang, Hu, Huang, & Wang, 2008), anti-tumor (Nie, Shi, & Ding, 2006), anticoagulation (Juliana, Elaine, Philip, & Marcello, 2002; Yang, Du, Huang, Wan, & Wen, 2005), immunoenhancement (Huang et al., 2008) and so on. Therefore, sulfated modification could be considered as the effective way to enhance the biological activities of polysaccharides. It is used for the Chinese herbal medicinal polysaccharide to improve its bioactivity as well (Tian, Li, Song, Zheng, & Li, 1995).

Lentinan (LNT) is a polysaccharide obtained from *Lentinus edodes* which is an edible mushroom popular in East Asia. It is well known as an anti-tumor agent (Oka et al., 1996) and immunostimulant (Kupfahl, Geginat, & Hof, 2006; Markova, Kussovski, & Drandarska, 2003) in China, Japan and Korea. However, whether sulfated modification could improve the biological activity of LNT or not? Sulfated LNT (sLNT) had strong anti-HIV activities, while whether it was also able to inhibit other virus or not?

In the present research, LNT was modified by chlorosulfonic acid–pyridine method with three modification conditions to obtain three sLNTs, $sLNT_1$, $sLNT_2$ and $sLNT_3$. The effects of three sLNTs on cellular infectivity of infectious bronchitis virus (IBV) for chicken embryo fibroblast (CEF) were compared by MTT method. The objective of this research was to investigate the probability of sulfating modification to improve anti-viral activity of LNT.

2. Materials and methods

2.1. Reagents

Eagle's minimum essential medium (MEM) (Gibco) supplemented with penicillin 100 IU/mL, streptomycin 100 IU/mL and 5% fetal bovine serum was called growth medium and used for culturing the cells, 2% fetal bovine serum, maintenance medium (MM) for diluting the polysaccharides and maintaining the cells. Hanks' solution was used for washing the chick embryo tissue shiver

Abbreviations: LNT, lentinan; sLNT, sulfated lentinan; IBV, infectious bronchitis virus; CEF, chicken embryo fibroblast; MEM, Eagle's minimum essential medium; MM, maintenance medium; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DS, degree of sulfation.

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and cells. Trypsin (Amresco-0858) was dissolved with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4) to 0.25%. 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved with CMF-PBS (pH 7.4) to 5 mg/mL. These reagents were filtered through a 0.22 μm filter. MEM and MM were stored at 4 °C, Trypsin solution, −20 °C, MTT solution, at 4 °C in dark bottles. Dimethyl sulfoxide (DMSO) was the production of Zheng-xing Institute of Chemical Engineering of Suzhou, Chlrosulfonic acid and pyridin, Sinopharm Chemical Reagent Co. Ltd. Other chemical used in experiment were analytical grade.

2.2. Extraction and purification of LNT

Lentinus edodes, bought from Fangge Company of Traditional Chinese Medicine, Zhejiang Province, was decocted with water into decoction. The crude total LNT (LNT_{tc}) was extracted from the decoction by ethanol precipitation whose content was 70% in the decoction.

LNTtc was purified as follows: to remove protein by Sevag's method (Zhang & Lu, 1999), to remove pigment by active carbon adsorption, then through D101 macroaperture resin column and G-200 Sephadex column in turn (Zhao, 1994). At last the purified LNT (LNT_{tp}) was obtained.

2.3. Sulfated modification of LNT

LNT was sulfated by the chlorosulfonic acid–pyridine method and the modified conditions were based on our preparative experiment (Chen, Wu, & Wang, 2005). In brief: Three chlorosulfonic acid–pyridine complex (1:2, 1:4, 1:6) were prepared in ice bath. Then, 400 mg LNT was added, respectively, stirred for 4 h at temperature 60 °C, dissolved in 100 mL ice-cold water, cooled to room temperature, neutralized with saturated NaOH solution and precipitated with 95% ethanol (EtOH) . The sediments were re-dissolved with water. The solution was dialyzed against tap water for 48 h and distilled water for 12 h in turn, then, lyophilized to obtain three sLNTs, $sLNT_1$, $sLNT_2$ and $sLNT_3$.

2.4. DS determination of sLNTs

The sulphur contents of three sLNTs were determined by Antonopoulos' method (Huang et al., 2008). 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 DSs of $sLNT_1$, $sLNT_2$ and $sLNT_3$ were 0.69, 0.98 and 1.37, respectively.

Three sLNTs, LNT_{tc} and LNT_{tp} were diluted with MM into fifteen concentrations from 3000 to 0.19 μg mL^{-1} in serial twofold. The diluted preparations were sterilized by pasteurization and stored at 4 °C for the test.

2.5. Cells and viruses

CEF were prepared with 10-day-old SPF chick embryo (Nanjing pharmaceutical & apparatus factory of China Animal Husbandry Industry Company). The cells was diluted into 1×10^6/mL with MEM and inoculated into 96-well culture plates (Nunclon) at 38.5 °C for 24 h in a humid atmosphere of 5% CO_2 for used.

IBV (H120 strain) was bought from Nanjing pharmaceutical & apparatus factory of China Animal Husbandry Industry Company. Its $TCID_{50}$ was 10^{-5} tested by Reed-Mueeh method. It was diluted into 10^{-3} (100 $TCID_{50}$) with MM for the test.

2.6. Determination of CEF safe concentration

When CEF became monolayers in 96-well plates, the series of concentrations polysaccharides at 3000, 1500, 750, 375, 187.5, 93.75, 46.88, 23.44, 11.72, 5.86, 2.93, 1.47, 0.74, 0.37 and 0.19 μg mL^{-1} were added into the plates, four wells each concentration. After a culture for 68 h at 38.5 °C in a humid atmosphere of 5% CO_2, 20 μl of MTT was added into each well, sequentially incubated for 4 h, 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 (Model DG-3022, East China Vacuum Tube Manufacturer).

A_{570} value is correlation to the number of live cells, the bigger A_{570} value is, the more live cells is. When A_{570} values of polysaccharide group were not significantly lower than that of cells control group, it indicated that the polysaccharides had not cytotoxicity, the corresponding concentrations were considered as maximal safety concentration for CEF.

2.7. Anti-viral assays

According to the tested result of safety concentration, five polysaccharide concentrations, from 5.86 to 0.19 μg mL^{-1}, were selected for determination of anti-viral activity by MTT method. When CEF cultured into monolayer, 5 polysaccharide dilutions at 5.86, 2.93, 1.47, 0.74, 0.37 and 0.19 μg mL^{-1} and IBV were added into cell plate, respectively, in three manners:

Pre-adding polysaccharide: firstly the polysaccharides solution were added into CEF plate, 100 mL/well and four wells per concentration. After incubated for 2 h at 38.5 °C in 5% CO_2, the polysaccharides solution were removed, the cell were washed twice with Hanks' solution and the virus solution was added.

Post-adding polysaccharide: firstly virus solution was added into CEF plate. After incubated for 2 h, virus solution was removed, the cell were washed twice with Hanks' solution and polysaccharides solution were added, four wells for each concentration.

Mixed adding polysaccharide with IBV: the polysaccharide solutions at each concentration were mixed with virus solution and incubated for 4 h at 4 °C, then added into CEF plate, four wells for each concentration.

All CEF plates were placed into 5% CO_2 incubator at 38.5 °C. When the IBV control group appeared obviously CPE (72 h), the CEF viability was measured by the MTT assay. The mean cellular A_{570} values were used as the indicator of anti-viral activity. When the A_{570} value of polysaccharide group was significantly higher than that of virus control group, it showed that the corresponding polysaccharide had significant anti-viral activity (Huang et al., 2008; Lu et al., 2008).

2.8. Statistical analysis

Data were expressed as means ± SD Duncan's multiple range test was used to determine the difference among polysaccharides and control groups. Differences between means were considered significant at $P < 0.05$.

3. Results

3.1. The maximal safe concentration

The CEF A_{570} values of every polysaccharide group were listed in Tables 1 and 2. In LNT_{tc} group, the CEF A_{570} values of 375–0.19 μg mL^{-1} groups were not significantly lower than that of cell

Table 1
The A_{570} value of each polysaccharide at 3000–23.44 μg mL^{-1}.

Concentration (μg mL^{-1})	LNT_t	LNT_1	$sLNT_1$	$sLNT_2$	$sLNT_3$
3000	0.18 ± 0.01^{d}	0.01 ± 0.01^{f}	0.02 ± 0.02^{e}	0.00 ± 0.00^{e}	0.01 ± 0.01^{d}
1500	0.22 ± 0.03^{c}	0.01 ± 0.01^{f}	0.14 ± 0.03^{d}	0.04 ± 0.01^{d}	0.11 ± 0.01^{d}
750	0.28 ± 0.02^{c}	0.13 ± 0.02^{e}	0.14 ± 0.02^{d}	0.07 ± 0.01^{d}	0.19 ± 0.03^{c}
375	0.58 ± 0.03^{a}	0.21 ± 0.02^{d}	0.15 ± 0.02^{d}	0.16 ± 0.02^{c}	0.24 ± 0.02^{c}
187.5	0.59 ± 0.03^{a}	0.34 ± 0.01^{c}	0.21 ± 0.01^{c}	0.23 ± 0.03^{b,c}	0.24 ± 0.01^{c}
93.75	0.57 ± 0.04^{a}	0.47 ± 0.02^{a,b}	0.28 ± 0.01^{c}	0.18 ± 0.01^{c}	0.35 ± 0.01^{b}
46.88	0.57 ± 0.02^{a}	0.50 ± 0.03^{a}	0.32 ± 0.01^{b,c}	0.24 ± 0.03^{b,c}	0.38 ± 0.01^{b}
23.44	0.56 ± 0.03^{a}	0.55 ± 0.02^{a}	0.41 ± 0.02^{b}	0.31 ± 0.02^{b}	0.37 ± 0.01^{b}
Cells control	0.53 ± 0.04^{a,b}	0.51 ± 0.02^{a}	0.51 ± 0.01^{a}	0.48 ± 0.02^{a}	0.54 ± 0.03^{a}

$^{a–f}$ Data within a column without the same superscripts differ significantly ($P < 0.05$).

Table 2
The A_{570} value of each polysaccharide at 11.72–0.19 μg mL^{-1}.

Concentration (μg mL^{-1})	LNTt	LNT1	sLNT1	sLNT2	sLNT3
11.72	0.56 ± 0.03^{a}	0.53 ± 0.03^{b,c}	0.37 ± 0.01^{b}	0.29 ± 0.02^{b}	0.40 ± 0.01^{c}
5.86	0.54 ± 0.02^{a}	0.57 ± 0.01^{b}	0.50 ± 0.03^{a}	0.49 ± 0.02^{a}	0.52 ± 0.02^{b}
2.93	0.48 ± 0.01^{a,b}	0.57 ± 0.01^{b}	0.51 ± 0.02^{a}	0.49 ± 0.02^{a}	0.62 ± 0.03^{a,b}
1.47	0.47 ± 0.03^{a,b}	0.54 ± 0.01^{b,c}	0.52 ± 0.04^{a}	0.52 ± 0.02^{a}	0.64 ± 0.03^{a}
0.74	0.47 ± 0.02^{a,b}	0.65 ± 0.01^{a}	0.52 ± 0.02^{a}	0.50 ± 0.02^{a}	0.66 ± 0.01^{a}
0.37	0.45 ± 0.04^{a,b}	0.65 ± 0.01^{a}	0.52 ± 0.01^{a}	0.54 ± 0.03^{a}	0.62 ± 0.02^{a,b}
0.19	0.45 ± 0.02^{a,b}	0.60 ± 0.03^{a,b}	0.50 ± 0.02^{a}	0.49 ± 0.03^{a}	0.68 ± 0.03^{a}
Cells control	0.53 ± 0.02^{a}	0.51 ± 0.02^{b,c}	0.51 ± 0.01^{a}	0.48 ± 0.02^{a}	0.54 ± 0.03^{b}

$^{a–c}$ Data within a column without the same superscripts differ significantly ($P < 0.05$).

control group. Its maximal safety concentration could be ascertained as 375 μg mL^{-1}. In LNT_{tp} group, the CEF A_{570} values of 93.75–0.19 μg·mL^{-1} groups were not significantly lower than those of cell control group. Its maximal safety concentration could be ascertained as 93.75 μg mL^{-1}. In $sLNT_1$, $sLNT_2$ and $sLNT_3$ groups, the CEF A_{570} values at 5.86–0.19 μg mL^{-1} groups were not significantly lower than those of cells control, so theirs maximal safety concentration could be determined as 5.86 μg mL^{-1}.

In order to compare their action at the same level, the maximal safety concentration of five polysaccharides were supposed as 5.86 μg mL^{-1}.

3.2. Anti-viral activities in pre-adding polysaccharide

The CEF A_{570} values of every group were listed in Fig. 1. The CEF A_{570} values of $sLNT_1$, $sLNT_2$ and $sLNT_3$ at all concentration groups were significantly larger than those of the virus control, and $sLNT_2$ group were significantly higher than those of other sLNTs groups ($P < 0.05$). The CEF A_{570} values of LNT_{tp} at 5.86, 2.93 and 1.47 μg mL^{-1} groups were significantly larger than those of the virus control ($P < 0.05$). The CEF A_{570} values of LNT_{tc} at 5.86 and 2.93 μg mL^{-1} groups were significantly larger than those of the virus control ($P < 0.05$).

3.3. Anti-viral activities in post-adding polysaccharide

The CEF A_{570} values of every group were listed in Fig. 2. The CEF A_{570} values of $sLNT_1$, $sLNT_2$ and $sLNT_3$ at 5.86, 2.93, 1.47, 0.74 and 0.37 μg mL^{-1} groups were significantly larger than those of the virus control, and $sLNT_2$ group were significantly higher than those of other sLNTs groups ($P < 0.05$). The CEF A_{570} values of LNT_{tp} at 5.86, 2.93, 1.47 and 0.74 μg mL^{-1} groups were significantly larger

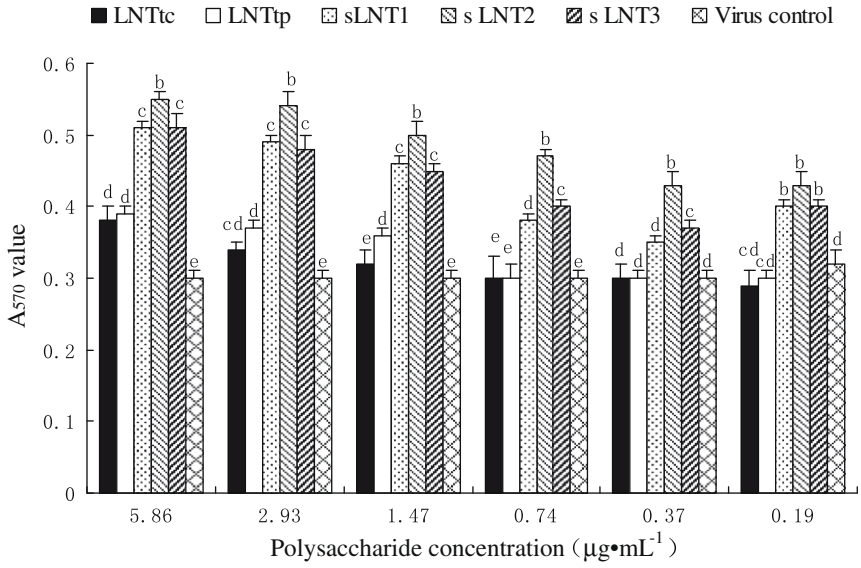

Fig. 1. Cellular A_{570} values of every group in pre-adding polysaccharide. $^{a–e}$Data within the same cluster of bars without the same superscripts differ significantly ($P < 0.05$).

than those of the virus control ($P < 0.05$). The CEF A_{570} values of LNT_{tc} at 5.86 μg mL^{-1} group was significantly larger than that of the virus control ($P < 0.05$).

3.4. Anti-viral activities in mixed adding with IBV

The A_{570} values of every group were listed in Fig. 3. The CEF A_{570} values of $sLNT_1$, $sLNT_2$ and LNT_{tp} at 5.86, 2.93 and 1.47 μg mL^{-1} groups were significantly higher than those of the virus control ($P < 0.05$) and $sLNT_2$ group were significantly higher than those of other sLNTs groups ($P < 0.05$). The CEF A_{570} values of $sLNT_3$ at 5.86 and 2.93 μg mL^{-1} groups were significantly higher than those of the virus control ($P < 0.05$). The CEF A_{570} values of LNT_{tp} at 5.86 μg mL^{-1}group was significantly higher than that of the virus control ($P < 0.05$).

4. Discussion

The safe concentration determination results showed that the maximal safety concentrations of three sLNTs were obviously lower than those of LNT_{tc} and LNT_{tp}. This indicated that sulfated modification could increase the cytotoxicity of lentinan. Other researches confirmed that the cytotoxicity of sulfated epimedium polysaccharide and sulfated astragalus polysaccharide were higher than that of non-sulfated epimedium polysaccharide and astragalus polysaccharide (Huang et al., 2008; Lu et al., 2008). But there were some natural sulfated polysaccharides with lower or without cytotoxicity (Mahmoud et al., 2002; Partha et al., 2004). The reason for this phenomenon was likely to be that synthesized sulfated polysaccharides had rudimental inorganic-sulfur or different structure. This should be noticed in application of sulfated polysaccharides.

The anti-viral experimental results showed that the anti-viral activities of three sLNTs were significantly stronger than those of non-sulfated LNT_{tp} and LNT_{tc}. For instance, in the manner of pre-adding polysaccharide (Fig. 1), non-sulfated LNT_{tp} and LNT_{tc} only at 5.86 and 2.93 μg mL^{-1} had significant anti-viral activity, while three $sLNT_S$ at all concentrations up to 0.37 μg mL^{-1} had significant anti-viral activity. In the manner of post-adding polysaccharide (Fig. 2), the A_{570} values of three sLNTs at 5.86–0.37 μg mL^{-1} were

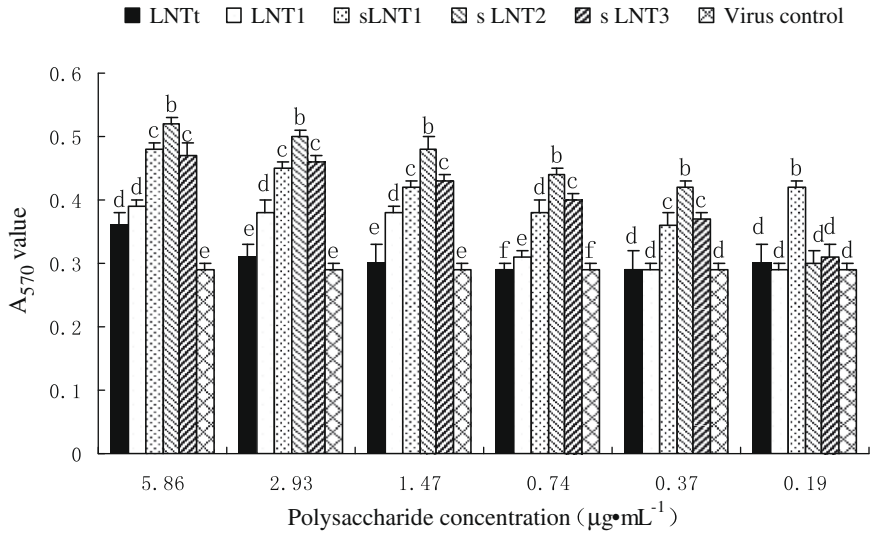

Fig. 2. Cellular A_{570} values of every group in post-adding polysaccharide. [a–f]Data within the same cluster of bars without the same superscripts differ significantly ($P < 0.05$).

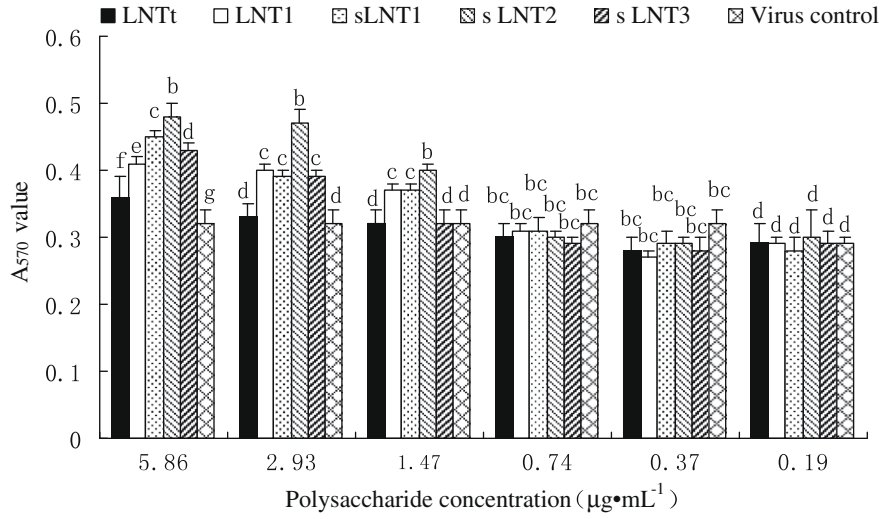

Fig. 3. Cellular A_{570} values of every group in mixed adding polysaccharides with IBV. [a–g]Data within the same cluster of bars without the same superscripts differ significantly ($P < 0.05$).

significantly larger than those of LNT_{tp} and LNT_{tc} ($P < 0.05$). In the manner of mixed adding (Fig. 3), the A_{570} value of three sLNTs at 5.86 μg mL^{-1} and $sLNT_2$ at 2.93, 1.47 μg mL^{-1} were significantly higher than those of LNT_{tp} and LNT_{tc} ($P < 0.05$). These confirmed that sulfated modification could enhance anti-viral activity of LNT. The sulfated modification researches of epimedium polysaccharide and astragalus polysaccharide also obtained the similar results (Zhang, Peter, Vincent, & Lina, 2004).

The activity of sulfated polysaccharide was closely related to the degree of sulfation (DS) (Alban, Schauerte, & Franz, 2002). In a certain scope, the higher DS was, the better biological activity was, but the activity could decrease when DS was too high. To make a comparison of three sLNTs, it could be seen that $sLNT_2$ possessed the strongest anti-viral activity, but it had not the hightest DS (0.98) being to middle between $sLNT_1$ (0.69) and $sLNT_3$ (1.37). This indicated that the DS of sulfated polysaccharides with the best anti-viral activity must was within optimum scope.

Since inhibitory effects of sulfated polysaccharides on viral attachments were reported more than 30 years ago (Masahiko et al., 1995), many researches reported the effects of sulfated polysaccharides, such as dextran sulfate, sulfated fungal β-glucans and so on, on several viruses, including human immunodeficiency virus (Liu, He, & Yang, 2004; Partha et al., 2004; Talarico et al., 2005; Zhang et al., 2004). *Lentinus edodes* has served as a model for investigating functional mushrooms and isolating pure compounds for pharmaceutical use (Iris, Dana, Shimona, Yitzhak, & Betty, 2006; Jong & Birmingham, 1993; Kupfahl et al., 2006). The present experiment also found the sLNTs could be stronger anti-viral activity comparison with LNT_{tp} and LNT_{tc}. However, the wide spectrum of activity of sulfated polysaccharides against various viruses makes them attractive for further development as candidate antiviral drugs (Masahiko et al. 1995).

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