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# Rapid and efficient microwave-assisted sulfate modification of lentinan and its antioxidant and antiproliferative activities in vitro

Yili Feng[a], Weiqi Li[a,*], Xueqian Wu[b], Liang He[b], Suyun Ma[a]

[a] Institute of Microbiology, College of Life Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, China
[b] Key Laboratory of Biological and Chemical Utilization of Forest Resources, Zhejiang Forestry Academy, Hangzhou 310023, China

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ABSTRACT

Lentinan (LNT) is a highly functional biomacromolecule, and its sulfated derivatives (sLNT) exhibit improved pharmacological properties compared to LNT. In the present study, LNT extracted from the fermentation broth of *Lentinus edodes* was sulfated using two methods, conventional heating and novel microwave radiation, and their biological activities were evaluated using an antioxidant system and an antiproliferation cell model. Our results indicate that the two sulfated derivates have similar physicochemical properties and spectroscopic profiles. Nevertheless, microwave radiation produced a higher yield of sLNT, with a shorter processing time and lower degradation. In contrast to LNT, the sLNT derivatives exhibited a strong antioxidant activity in vitro. In addition, the derivatives exhibited a significant antiproliferative effect on leukemia cells, whereas LNT exhibited the opposite outcome.



## 1. Introduction

Presently, much interest has been focused on polysaccharides produced by a vast number of different fungi. These polysaccharides have important biological properties, such as immunostimulatory and antitumor effects, which have been exploited by the healthcare, food and pharmaceutical industries (Pokhrel & Ohga, 2007; He et al., 2009; Sun, Liang, et al., 2009). The water-soluble polysaccharide lentinan (LNT), which is a β-glucan, is the most important macromolecule isolated from *Lentinus edodes*. This polysaccharide has immunomodulatory (Ruan, Su, Dai, & Wu, 2005), antitumor (Surenjav, Zhang, Xu, Zhang, & Zeng, 2006), antinociceptive and antiinflammatory (Carbonero et al., 2008) activities. In addition, while it does not exhibit direct cytotoxic effects against tumor cells, LNT has been shown to effectively mediate antitumor immune responses (Guo et al., 2009; Suzuki, Kikuchi, Takatsuki, & Hamuro, 1994).

Four main factors can influence the bioactivity of a polysaccharide: water solubility, molecular weight ($M_w$), chain conformation and introduction of suitable ionic groups with an appropriate degree of substitution (DS) (Alban & Franz, 2001; Sun, Liang, et al., 2009; Yang, Du, Wen, Li, & Hu, 2003). Chemically modifying polysaccharides, such as by sulfation (Bao, Zhen, Ruan, & Fang, 2002), phosphorylation (Dace et al., 1997), methylation (Singh, Tiwari, Tripathi, & Malviya, 2003), or carboxymethylation (Bao et al., 2002), can result in the formation of new pharmacological agents with therapeutic applicability.

The antiviral activity of sulfated polysaccharides was reported in the late 1980s (Witvrouw & DeClercq, 1997). Today, sulfation is the most widely used and effective way to enhance the biological activities of polysaccharides; the resulting compounds, which are highly water-soluble and exist in a metabolized form, have been shown to have minimal toxicity (Raghuraman, Riaz, Hindle, & Desai, 2007). However, unlike the well-studied endopolysaccharides produced by the fruiting bodies of medicinal fungi, studies on the exo- and endopolysaccharides of the submerged mycelia are at their early stages (Scherba & Babitskaya, 2008). This is due in part to the lack of the required biotechnology for the development of submerged fermentation to obtain polysaccharides with biological properties.

Free radicals and their derivatives, which are produced during metabolism or potential environmental impact, induce the damage of biomolecules and promote life-threatening health problems, such as cardiovascular diseases (Gey, 1993), diabetes (Xue, Chen, Lu, & Jin, 2009), arthritis (Biemond, Swaak, & Koster, 1984), cancer (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970), and aging (Zhang et al., 2003). Although living organisms possess antioxidant mechanisms that protect them from such damage, an increased

*Abbreviations:* LNT, lentinan; sLNT, sulfated lentinan; $M_w$, molecular weight; DS, degree of substitution; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; EPS, exopolysaccharide; CSA, chlorosulfonic acid; Pyr, pyridine; HPSEC, high performance size-exclusion chromatography; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

* Corresponding author. Tel.: +86 0571 88206629.
*E-mail addresses:* liweiqi2007@zju.edu.cn, weiqili01@gmail.com (W. Li).

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**Table 1**
Characterization of LNT and its sulfated derivatives.

| Sample | Yield (mg) | Carbohydrate content[a] | DS[b] | $10^6$ $M_w$[c] (Da) | $M_w/M_n$[d] |
|---|---|---|---|---|---|
| LNT | n.d.[e] | 97.2 | n.d.[e] | 1.55 | 1.25 |
| sLNT-1 | 140 | 58.8 | 1.45 | 1.01 | 1.65 |
| | | | | 1.84 | 1.27 |
| sLNT-2 | 180 | 49.6 | 1.57 | 1.62 | 1.44 |

[a] Carbohydrate content is expressed as dry weight (%).
[b] Number of sulfate groups per sugar unit (DS = degree of substitution) as determined using the barium chloride-gelatin method and calculated according to the following formula: $DS = (162 \times S\%)/(32 - 102 \times S\%)$.
[c] Average molecular weight determined by HPSEC (mobile phase, double-distilled water, using dextran as a reference).
[d] Represents the molecular weight distribution.
[e] Not determined.

load of free radicals and an inadequate antioxidant defense mechanism can result in increased levels of lipid peroxide, which can lead to the mutagenesis and carcinogenesis. However, polysaccharides and their derivatives can change the expression of signals within tumor cells (Zhang, Cui, Cheung, & Wang, 2006), where free residuals can act as second messengers in intracellular signaling cascades and induction of a mitogenic response (Valko et al., 2007). Sulfated polysaccharide derivatives may exert their antiproliferative effects by suppressing metastasis and trapping superoxide radicals that promote the progression of malignant tumor cells (Ooi & Liu, 2000). Studies have shown that, due to their antioxidant properties, polysaccharides can decrease the viability of leukemia cells and induce apoptosis; however, these studies have shown that most polysaccharides are protein-bound (Athukorala, Kim, & Jeon, 2006; Chen, Wang, & Wu, 2009; Otagiri, Ohkuma, Ikekawa, & Tanaka, 1983; Wong, Wong, Chiu, & Cheung, 2007).

In the present study, LNT was isolated and purified from the fermentation broth of *L. edodes* using mild extraction steps, thus preventing its macromolecular chain depolymerization and biological inactivation. In addition, we generated sulfated derivatives of LNT (sLNT) by conventional heating and by using a novel microwave radiation method. Furthermore, we studied their physicochemical properties of these derivatives, examined their antioxidant activity in vitro and assessed their antiproliferative effects against leukemia cells.

## 2. Materials and methods

### 2.1. Biological material

To produce LNT, *L. edodes* (ZFA0033) was cultured in a 100-L airlift fermenter as previously described (Feng, Li, Wu, Cheng, & Ma, 2010).

The human leukemia cell line HL-60 was obtained from the Institute of Biochemistry of Zhejiang University. The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in an environment of 5% $CO_2$. 180 μL of this cell suspension were seeded into each well of a 96-well plate which was then incubated at 37 °C.

### 2.2. Extraction and purification of the exopolysaccharide (EPS)

50 L of fermentation broth were concentrated to 5 L in a rotary evaporator below 45 °C and left to cool. Then, to precipitate the polysaccharide, an excess of 95% ethanol (4/1; v/v) was added, and the mixture was incubated at 4 °C for 12 h. The precipitate was resuspended in an equal volume of 75% ethanol to remove oligosaccharides. The EPS was collected by centrifugation at 11,712 × *g* for 30 min at 4 °C and was dissolved in water to remove insoluble substances. The soluble fraction was deproteinated using the Sevag method (Staub, 1965) more than ten times until spectroscopic analysis in the UV spectrum (UV-3150 SHIMADZU, Japan) indicated that the solution had no absorption at 260- and 280-nm wavelength. After the addition of 30% $H_2O_2$ to discolor, the solution was dialyzed against tap water for 3 days and against distilled water for an additional 3 days at relative low room temperature ($M_w$ cutoff = 8–10 kDa). The resulting polysaccharide solution was concentrated in a rotary evaporator at reduced pressure and at a temperature below 45 °C, and then lyophilized to obtain the crude exopolysaccharide in the form of a colorless powder.

The powder was then dissolved in distilled water, which formed cold water-soluble and -insoluble fractions, and the latter was separated by filtering through a 0.45-μm pore filter (Millipore, USA). The soluble fraction was further purified by ultrafiltration through a 300-kDa cut-off membrane (Biomax-300) supported on a filter holder (Millipore, USA) to obtain the retentate and eluate. The retentate was further diluted and purified by ultrafiltration three times and was then lyophilized.

### 2.3. Sulfate modification of the polysaccharide

The sulfation reagent, which is a complex of chlorosulfonic acid (CSA) and pyridine (Pyr), was prepared by slowly adding 5 mL of CSA to 25 mL of Pyr in a three-necked flask with continuous stirring and cooling in a 0–4 °C ice bath.

#### 2.3.1. Sulfation by conventional heating

Chemical sulfation of LNT was carried out by using the CSA-Pyr method described by Yoshida et al. (1995). Briefly, 150 mg of LNT were resuspended in 20 mL of dry formamide, and the sulfation reagent was added stepwise. The mixture was maintained at 45 °C for 6 h and stirred with a magnetic stir bar. After the reaction ended, the mixture was cooled and neutralized with a solution of 2.5 M NaOH then precipitated by 95% ethanol. Finally, the sulfated polysaccharide was dialyzed against distilled water and lyophilized. LNT sulfated using this method is referred to as sLNT-1.

#### 2.3.2. Sulfation by microwave radiation

The microwave-assisted sulfation was placed in the center of a microwave oven turn table and exposed to a power of 100 W for 1.5 min. The reaction was then cooled in an ice water bath to avoid thermal degradation of the reaction due to overheating. This heating and cooling process was repeated 3 times. The resulting mixture was then cooled, neutralized with NaOH, precipitated by ethanol, dialyzed and lyophilized as described above. LNT sulfated using this method is referred to as sLNT-2.

### 2.4. Characterization of LNT and sLNT

Homogeneity and molecular weight determinations were made using a high performance size-exclusion chromatography (HPSEC) instrument (Waters 2410, USA) equipped with a differential refractive index detector. Briefly, the polysaccharide solutions were

individually loaded onto Ultrahydrogel 2000 (7.8 mm × 300 mm) and Ultrahydrogel 250 (7.8 mm × 300 mm) columns and were run at a flow rate of 0.7 mL/min at 40 °C using double-distilled water as the mobile phase. The solutions were filtered through 0.45-μm filters (Millipore, USA) prior to injection, and 10 μL (10 mg/mL) of solution were analyzed for 30 min in each injection. The columns were calibrated using standard T-series dextrans (T-2000, T-500, T-200, T-80, and T-10).

The carbohydrate content of the polysaccharides was measured using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The DS for each derivative was determined using the barium chloride-gelatin assay, and potassium sulfate was used as the standard (Dodgson & Price, 1962). The FT-IR spectra of the polysaccharides were recorded using the KBr disc on a Nicolet Nexus-670 (USA) instrument in the range of 4000–400 $cm^{-1}$, with 1024 scans for each spectrum. The $^{13}C$ NMR spectra of 80 mg/mL solutions in $D_2O$ were recorded on a Bruker AVIII (Switzerland) instrument at 500 MHz at 40 °C.

### 2.5. Antioxidant activities of LNT and sLNT in vitro

To assess the antioxidant activity of the polysaccharides, we examined the following four aspects: superoxide radical scavenging capacity, hydroxyl radical scavenging activity, lipid antioxidant capacity and ferrous ion ($Fe^{2+}$) chelation.

Superoxide radical scavenging activity was measured using commercially available biochemical kits (Nanjing Jiancheng Bioengineering Institute, China). Briefly, superoxide radicals were generated using the xanthine–xanthine oxidase system, with adding of polysaccharide solutions at varying final concentrations (10, 25, 50, 100, 200 and 400 μg/mL) and measuring their absorbance at 550 nm using the Griess reagent.

Hydroxyl radical scavenging activity was measured using commercially available biochemical kits (Nanjing Jiancheng Bioengineering Institute, China). Briefly, hydroxyl radicals were generated using the Fenton system, with adding of polysaccharide solutions at varying final concentrations (10, 25, 50, 100, 200 and 400 μg/mL) and measuring their absorbance at 550 nm using the Griess reagent.

The condensation of the lipid peroxide decomposition products malondialdehyde (MDA) and thiobarbituric acid was used to monitor lipid peroxidation by using egg yolk homogenates as the oxidation medium (Dasgupta & De, 2004). Briefly, 0.4 mL of egg homogenate (10%, v/v) and 0.1 mL of polysaccharide solutions at varying final concentrations (10, 25, 50, 100, 200 and 400 μg/mL) were mixed and brought up to a volume of 1 mL with distilled water; these reactions were then incubated at 37 °C for 30 min. After adding 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulfate, the mixtures were vortexed and heated at 95 °C for 60 min until a pink color appeared. The mixtures were then cooled under circulating water, mixed with 5 mL of n-butanol and centrifuged at 5856 × *g* for 10 min. Finally, the absorbance of the fractionated organic layer at 532 nm was measured.

$Fe^{2+}$ chelation was measured as previously reported (Dinis, Madeira, & Almeida, 1994). Briefly, 3.7 mL of polysaccharide solutions at varying final concentrations (0.25, 0.5, 1, and 2 mg/mL) were reacted with 0.1 mL of 2 mM $FeCl_2$ for 30 s, after which 0.2 mL of 5 mM ferrozine were added. The mixture was left to stand for 10 min, and the absorbance of the mixture at 562 nm was measured.

### 2.6. Growth inhibition of leukemia cells

After culturing the leukemia cells for 16 h, 20 μL of LNT and sLNT were added to the cultures to give final concentrations of 25, 50, 100, 200, 300, and 400 μg/mL, and the cells were incubated for 72 h. Cell proliferation was measured using the MTT assay (Athukorala et al., 2006). For this purpose, 50 μL of 0.4% 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were added to the cells, which were then incubated for another 4 h. The cells were centrifuged at 600 × *g* for 5 min. The supernatants were removed, and 150 μL of dimethyl sulfoxide (DMSO) were added to terminate the reaction. The absorbance of the cells at 570 nm was then measured using a multiwell plate reader.

## 3. Results and discussion

### 3.1. The characterization of LNT and its modified derivatives

HPSEC revealed that LNT was homogeneous. Based on its retention time, we estimated that LNT had a molecular weight of $1.55 \times 10^6$ Da (Table 1). In our experiments, it was important to maintain the macromolecular features of the modified derivatives because they can degrade during the sulfation process. CSA is the most widely used sulfation reagent; however, the high acidity of CSA can cause the cleavage of labile functional groups and backbone depolymerization. Although Pyr has been employed as an aprotic reaction solvent to make the reaction mixture more homogeneous while maintain a high degree of polymerization (Papy-Garcia et al., 2005), the depolymerization is inevitable. Thus, decreasing the amount of exposure of the polysaccharides to the acidic reagent becomes an alternative choice.

Fractionation of sLNT-1 produced two compounds with $M_w$ of $1.01 \times 10^6$ and $1.84 \times 10^6$ Da, respectively. In contrast, fractionation of sLNT-2 resulted in a single homogenous compound with a $M_w$ of $1.62 \times 10^6$ Da (Table 1). Sulfation of LNT using microwave radiation led to a narrower $M_w$ distribution of derivative. The DS of sLNT-1 and sLNT-2 was of 1.45 and 1.57, respectively.

Theoretically, we expected a pronounced increase in the $M_w$ of LNT upon sulfation. However, neither sLNT-1 nor sLNT-2 exhibited a significant $M_w$ increase, which indicated that the backbones of these modified polysaccharides were partially degraded, resulting in a relative higher $M_w/M_n$ than for the precursor. Several studies have reported the use of microwave radiation for the acceleration of organic reactions using polar solvents in organic chemistry, but it has been strictly employed in carbohydrate chemistry due to the biological properties of polysaccharides. Moreover, some studies have reported that microwave radiation results in desulfated and low molecular-weight products (Navarro, Flores, & Stortz, 2007; Xing et al., 2004). However, microwave radiation does not cleave specific bonds in molecules and requires a short exposure time because the ionic sulfated product may couple to the microwaves through ionic conduction (Raghuraman et al., 2007). Herein, the mild reaction conditions and intermittent treatment resulted in a high DS for the modified polysaccharides, significantly increasing their yield and decreasing their degradation. It is important to note, however, that degradation is an inevitable consequence of the reaction due to the high reagent to the sugar unit ratio (Sun, He, et al., 2009) and its high acidity. The non-uniform heating conditions in the conventional system caused the formation of a non homogeneous product, despite the continuous mixing during the course of the reaction, because energy is transferred through the solvent by thermal conduction. The reduced reaction time can also cause less solvolysis in formamide, leading to an enhanced rate, yield and purity.

Fig. 1 shows the FT-IR absorption spectra of LNT and its modified derivates with bands at 4000–400 $cm^{-1}$. The typical signals of the polysaccharides were exhibited at 1650, 1400, 1060 and 977 $cm^{-1}$. The FT-IR spectra of the sLNTs show that the OH stretching vibration band was narrowed. Additionally, sLNTs display two characteristic absorptive bands at 1262/1262 $cm^{-1}$ (S=O) and 816/815 $cm^{-1}$

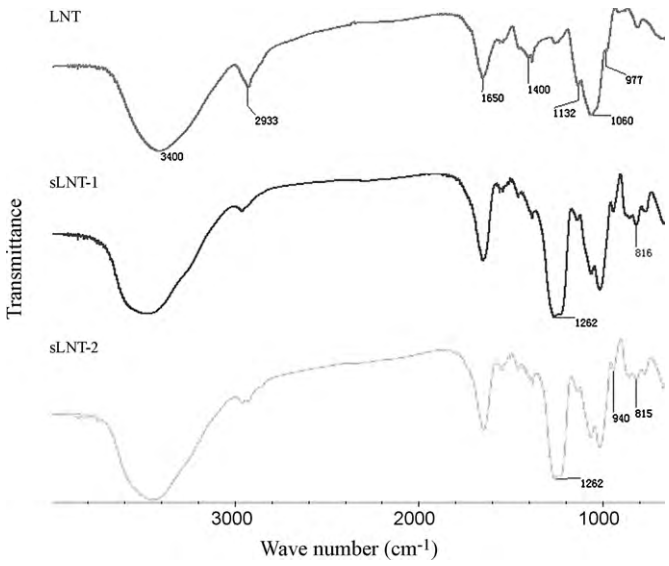


**Fig. 1.** FT-IR spectra of LNT and its sulfated derivatives on KBr disks.

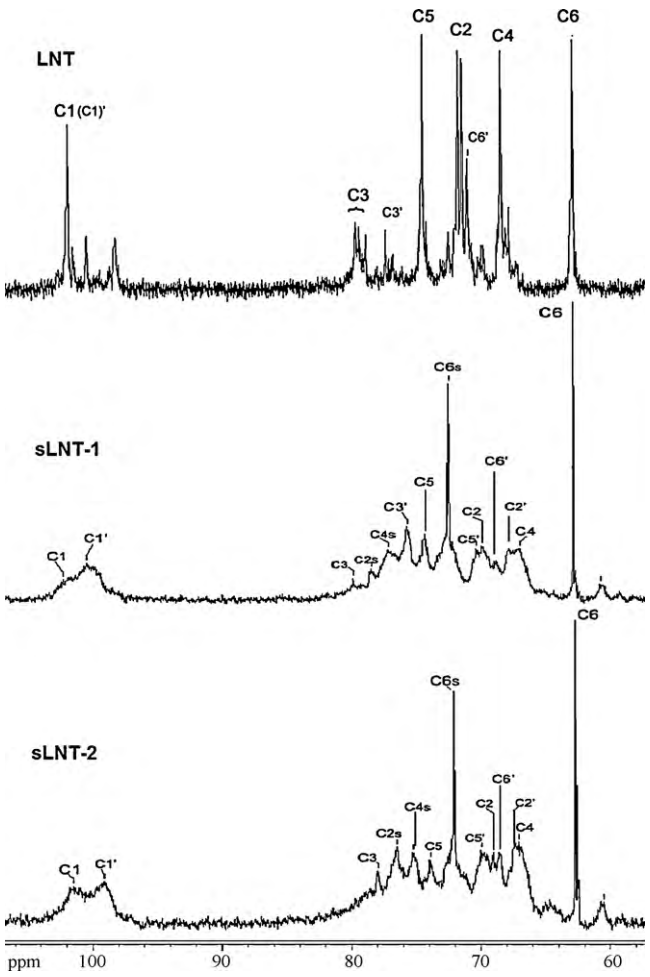


**Fig. 2.** $^{13}C$ NMR spectra (500 MHz) of LNT and its sulfated derivatives in $D_2O$ (80 mg/mL) at 40 °C. The "-s" denotes a shift caused by sulfation of a hydroxyl group on the carbon. The "-" denotes a shift influenced by substitution of an adjacent carbon.

(C–O–S). These results indicate that the sulfate groups were appropriately incorporated into the polysaccharides, thus forming sulfate esters (Bao et al., 2002; Ghosh et al., 2004). The band at 940 $cm^{-1}$ corresponds to the pyranose units of the polysaccharide and proves that the cyclic pyranosyl rings were not destroyed by microwave radiation.

NMR spectroscopy is an especially powerful method for the full structural characterization of a polysaccharide. Due to the high $M_w$ of the polysaccharides, it was difficult for us to obtain idealized NMR spectra that could be used for the proper interpretations of the shifts, even though we used a high testing concentration of samples with increasing scan number.

Fig. 2 shows the $^{13}C$ NMR spectrum of LNT and its derivatives. The signals at C1 (102.3), C2 (70.4), C3 (78.7), C4 (66.9), C5 (73.3), and C6 (61.1) corresponded to LNT. C1′ (102.2), C3′ (76.3) and C6′ (69.8) corresponded to the substituted side residues, which indicated that LNT consists of a (1→3) linked backbone bound to a (1→6) branch. The multiplicity and broadness of the C3 signal is due to the presence of linear (1→3), branched (1→6) and terminal glucosyl residues on LNT (Wang & Zhang, 2009).

The distribution of the introduced sulfate residues was determined by NMR. The carbons that are directly attached to an electron-withdrawing sulfate group exhibit strong downfield (α-effect) and upfield shifts with respect to the adjacent carbon (β-effect).

The spectrum of sLNT-1 was more complicated (Fig. 2). The new peak at C6s (72.4) represents the signal of the O6-substituted carbon, and the decreased intensity of the C6 peak suggests that the OH groups on the internal side of the helix chain were not completely sulfated. The new peaks at C2s (69.8) and C4s (67.0) corresponded to the partial O2 and O4 substitutions. These peaks appeared due to the α-effect, and the downfield shift of the carbon directly linked to the sulfate group was of approximately 7–10 ppm. The range of the shift due to the electron density, which is influenced by the intermolecular hydrogen bonds and the interaction between the charged groups, caused cationation (Zhang, Zhang, Zhou, Chen, & Zeng, 2000). The new C1′ (99.7) peak that split from the anomeric region of C1 was influenced by the substitution of the adjacent C2. The C2′ (67.8) and C5′ (70.3) peaks resulted from the substitution of adjacent carbons of C3 and C6, respectively. The appearance of these peaks corresponds with the influence of the β-effect.

The spectrum of sLNT-2 was similar to that of sLNT-1. The sulfate groups were not evenly distributed throughout the polysaccharide chain, but rather, they were non-selectively substituted. It is important to note that the intensity of the C6s peak in the two derivatives was relatively stronger than that of the C2s and C4s peaks. This difference is due to low steric hindrance and weak electrostatic repulsions, which indicates that the C6 substitution predominated during the entire sulfation process. The intensity of the C6s peak in the spectrum of sLNT-2 is weaker than that for sLNT-1, but the intensity of C2s and C4s are stronger for sLNT-2 (data not shown). Although not large, this difference is real, indicating that microwave radiation reduces steric hindrance and maintains LNT in an ordered, extended structure during the reaction. The overlap of the signals for sLNT-2 was greater than for sLNT-1, because the electronic environment and the advanced structure of sLNT-2 became more complex after microwave radiation. The complexity of the structure of sLNT-2 was confirmed by the increased DS obtained from the data calculation. Notably, the lack of signals at 125–150 ppm (data not shown) indicated that there was no introduction of pyridinium, which is cytotoxic to normal cells (Petit et al., 2004).

### 3.2. Antioxidant activity of LNT and sLNT in vitro

Superoxide radicals are the first oxygen radicals produced in vivo and further produce various kinds of oxidizing agents that propagate free radical chain reactions. The hydroxyl radical is the most active free radical that attacks all biomolecules (Navarro et

**Table 2**
Superoxide radical scavenging activity (%) of LNT and its sulfated derivatives at different concentrations.[a].

| Sample | Concentration (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 10 | 25 | 50 | 100 | 200 | 400 |
| LNT | −0.25 ± 1.74 | −5.64 ± 1.39 | −6.01 ± 3.82 | −4.79 ± 2.08 | −0.92 ± 1.30 | 3.19 ± 0.17 |
| sLNT-1 | −3.13 ± 0.26 | 24.05 ± 1.21** | 31.47 ± 0.95** | 41.41 ± 0.26** | 45.46 ± 1.30** | 47.36 ± 0.35** |
| sLNT-2 | 1.66 ± 0.95 | 26.69 ± 0.78** | 39.39 ± 1.04** | 42.76 ± 2.34** | 48.96 ± 0.69** | 48.10 ± 2.60** |

[a] The % superoxide radical scavenging activity was calculated using the following formula: $[(A550_{blank} - A550_{sample})/A550_{blank}] \times 100\%$. All experiments were carried out in triplicate, and the values shown represent the means of triplicates ± SD, and ascorbic acid (150 μg/mL) was used as the positive control (42.64 ± 1.99%).
** Significant difference from the LNT was evaluated using *t*-test: $P < 0.01$.

al., 2007; Xing et al., 2004). The genotoxic products of free radical-induced lipid peroxidation have unpaired electrons, which can attract electrons from other molecules, causing damage to DNA by forming endogenous DNA adducts (Burcham, 1998; Zhu et al., 2004). Production of lipid-derived free radicals is an early oxidative event in murine leukemia cells due to photosensitization (Kelley, Buettner, & Burns, 1997). Most free radical-induced injury is iron-related. Iron catalyzes biological free radical oxidation and induces the formation of free radicals and lipid peroxides (Reizenstein, 1991).

Antioxidants can scavenge harmful free radicals, which are potentially toxic to various biomolecules due to their ability to promote lipid peroxidation, DNA damage and inhibition of protein synthesis (Zhao et al., 2004), and can protect cells from mutations. The antioxidant activity of polysaccharides has been well reported (Zhang et al., 2003), but only recently have sulfated polysaccharides (from a marine fungus) been shown to possess such enhancement (Yang, Gao, Han, & Tan, 2005). We thoroughly evaluated the antioxidant properties of LNT and sLNT based on their superoxide radical scavenging capacity, hydroxyl radical scavenging activity, lipid antioxidant capacity and $Fe^{2+}$ chelation. Overall, our results indicated that the incorporation of a sulfate group into the structure of LNT significantly increases its antioxidant activity in vitro.

### 3.2.1. Superoxide radical scavenging activity

In biological systems, superoxide radicals are a by-product of the normal catalytic processes of certain enzymes. Although superoxide radicals are weak oxidants, they can induce the production of hydroxyl radicals and lipid peroxidation. The sLNTs that we generated exhibited strong scavenging activity against superoxide anion radicals in a dose-dependent manner. In contrast, LNT did not have such ability. The scavenging activities of sLNT-1 and sLNT-2 at a concentration of 400 μg/mL were 47.4% and 48.1%, respectively (Table 2).

### 3.2.2. Hydroxyl radicals scavenging activity

Both sulfated derivatives displayed a strong, dose-dependent hydroxyl radical scavenging activity, while unsulfated LNT displayed a low activity. The hydroxyl radical scavenging activity of sLNT-1 and sLNT-2 at a concentration of 400 μg/mL reached 68.25% and 71.58%, respectively (Table 3). These results indicated that sulfation of LNT strongly influences its ability to scavenge reactive oxygen species.

### 3.2.3. Lipid peroxidation assay

Lipid peroxides are likely involved in numerous pathological events. For example, lipid peroxides destroy membrane lipids, resulting in the production of breakdown products, such as MDA, which are released from the cells in microsomes (Burcham, 1998). The production of MDA is an important indicator of endogenous lipid peroxidation.

The sulfated derivatives exhibited a strong ability to inhibit the activity of lipid peroxides. The inhibition rate of sLNT-1 and sLNT-2 at a concentration of 400 μg/mL reached at 46.40% and 54.94%, respectively, while unsulfated LNT exhibited only a low inhibitory capacity (Table 4).

### 3.2.4. $Fe^{2+}$ chelation

Superoxide radicals can induce the release of iron from ferritin. In turn, iron induces the formation of free radicals by catalyzing

**Table 3**
Hydroxyl radical scavenging activity (%) of LNT and its sulfated derivatives at different concentrations.[a].

| Sample | Concentration (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 10 | 25 | 50 | 100 | 200 | 400 |
| LNT | 4.35 ± 2.56 | 4.40 ± 0.28 | 5.58 ± 1.94 | 5.58 ± 2.49 | 9.20 ± 0.55 | 12.48 ± 0.21 |
| sLNT-1 | 7.53 ± 0.83 | 15.07 ± 1.11** | 29.26 ± 2.08** | 53.13 ± 0.14** | 65.80 ± 0.35** | 68.25 ± 0.48** |
| sLNT-2 | 8.90 ± 3.32 | 16.73 ± 0.42** | 35.52 ± 0.14** | 56.75 ± 1.11** | 68.69 ± 0.42** | 71.58 ± 0.62** |

[a] The % hydroxyl radical scavenging activity was calculated using the following formula: $[(A550_{blank} - A550_{sample})/A550_{blank}] \times 100\%$. All experiments were carried out in triplicate, and the values shown represent the means of triplicates ± SD, and ascorbic acid (150 μg/mL) was used as the positive control (76.54 ± 1.32%).
** Significant difference from the LNT was evaluated using *t*-test: $P < 0.01$.

**Table 4**
Lipid peroxide inhibition (%) of LNT and its sulfated derivatives different concentrations.[a].

| Sample | Concentration (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 10 | 25 | 50 | 100 | 200 | 400 |
| LNT | 3.93 ± 1.75 | 8.76 ± 1.27 | 10.11 ± 1.59 | 9.44 ± 2.22 | 12.58 ± 0.64 | 11.91 ± 3.18 |
| sLNT-1 | 18.76 ± 3.34* | 28.20 ± 5.24* | 31.69 ± 4.77* | 41.35 ± 2.86** | 42.92 ± 3.50** | 46.40 ± 2.07** |
| sLNT-2 | 15.28 ± 2.86* | 30.34 ± 2.54** | 37.19 ± 2.70** | 42.81 ± 2.07** | 51.57 ± 1.75** | 54.94 ± 1.43** |

[a] The % lipid peroxide inhibition activity was calculated using the following formula: $[(A532_{blank} - A532_{sample})/A532_{blank}] \times 100\%$. All experiments were carried out in triplicate, and the values shown represent the means of triplicates ± SD and ascorbic acid (150 μg/mL) was used as the positive control (45.65 ± 2.35%).
* Significant difference from the LNT was evaluated using *t*-test: $P < 0.05$.
** Significant difference from the LNT was evaluated using *t*-test: $P < 0.01$.

**Table 5**
$Fe^{2+}$ chelating ability (%) of LNT and its sulfated derivatives at different concentrations.[a].

| Sample | Concentration (mg/mL) | | | |
|---|---|---|---|---|
| | 0.25 | 0.5 | 1.0 | 2.0 |
| LNT | 16.94 ± 1.25 | 15.53 ± 2.94 | 17.65 ± 0.68 | 18.12 ± 1.21 |
| sLNT-1 | 18.59 ± 0.68 | 16.00 ± 1.26 | 17.88 ± 2.30 | 20.12 ± 0.75 |
| sLNT-2 | 19.29 ± 1.85 | 22.59 ± 1.11 | 31.53 ± 0.98** | 36.76 ± 0.69** |

[a] The % chelating ability was calculated using the following formula: $[(A562_{blank} - A562_{sample})/A562_{blank}] \times 100\%$. All experiments were carried out in triplicate, and the values shown represent the means of triplicates ± SD, and EDTA (10 μg/mL) was used as the positive control (31.76 ± 1.05%).
** Significant difference from the LNT was evaluated using *t*-test: $P < 0.01$.

the conversion of superoxide radicals into toxic hydroxyl radicals (Herbert, Shaw, Jayatilleke, & Stoplerkasdan, 1994; Reizenstein, 1991).

LNT showed a relatively moderate ability to chelate $Fe^{2+}$, while substitution of its hydroxyl group with sulfate increased this ability. In particular, the $Fe^{2+}$ chelation ability of sLNT-2 significantly increased in a dose-dependent manner evaluated by *t*-test (Table 5).

Among various antioxidant molecules, sulfated polysaccharides can scavenge free radicals, bind metal ion catalysts to prevent the continuous production of radicals, and prevent lipid peroxidation to the propagation of free radicals. Polysaccharides exert their antioxidant activity by abstraction of the anomeric hydrogen from the internal monosaccharide units (Athukorala et al., 2006). Sulfated polysaccharides have a stronger antioxidant activity, which is partly due to their ordered, extended structure; their buried active groups, which are exposed by the introduction of sulfate groups; and their improved solubility after sulfate substitution. Sulfated polysaccharides usually trap free radicals in an electrostatic manner because the sulfate groups produce a highly acidic environment. The sulfur substitution may weaken hydrogen bond interactions between polysaccharides. Our results indicated that unsulfated LNT has nearly undetectable antioxidant activity, while its sulfated derivatives exhibit a significant level of such activity that is likely due to the introduction of the sulfate groups.

### 3.3. Inhibition of leukemia cell growth by LNT and sLNT

Free radicals partly contribute to the leukemogenesis. In addition, free radicals promote oxidative damage and metastasis (Alcain, Buron, Rodriguezaguilera, Villalba, & Navas, 1990; Devi et al., 2000; Subrahmanyam, Ross, Eastmond, & Smith, 1991). An efficient antioxidant, such as sLNT, can reduce the oxidant activities of such radicals.

Currently, the inhibitory effects of natural products are evaluated by testing their ability to inhibit the proliferation of the cancer cells directly (Athukorala et al., 2006), or by determining their ability to induce immune cells to secrete cytokines, which act on the cancer cells (Chen & Chang, 2004). In this study, we found that sLNT exhibited strong antioxidant activity, which in turn directly inhibited the growth of leukemia cells.

HL-60 cells were treated with increasing concentrations (25–400 μg/mL) of LNT or sLNTs. Fig. 3 shows the in vitro proliferation inhibition of the different polysaccharides. LNT did not significantly inhibit even contribute to the growth of the leukemia cells. In contrast, sLNT significantly inhibited the growth of these cells. The maximum inhibitory effect for sLNT-1 and sLNT-2 (27.45% and 38.59%, respectively) was observed when the cells were treated with 200 μg/mL of the polysaccharides. In addition, we found that sLNT-2 was more effective than sLNT-1 at the concentration of 100 μg/mL. Although this does not represent a significant difference at all the concentrations assayed, the difference does show a

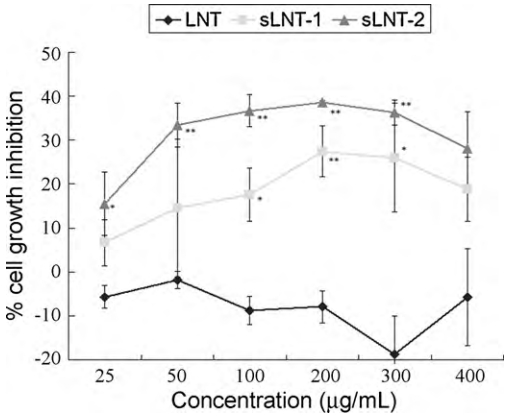


**Fig. 3.** Dose-dependent ability of LNT and its sulfated derivatives to inhibit the proliferation of HL-60 cells. The % inhibition was calculated using the following formula: $[(A570_{blank} - A570_{sample})/A570_{blank}] \times 100\%$. All experiments were carried out in triplicate, and the values shown represent the means of triplicates ± SD. Significant differences from the LNT were evaluated using *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

trend that can be attributed to microwave radiation, which likely increased the DS of the substituted C2 and C4 in sLNT-2, resulting in a structure with high inhibitory activity.

Many studies have reported that unmodified polysaccharides, most of which are glycoproteins, can decrease the viability of leukemia cells and inhibit their proliferation and migration. In contrast to Wang's report (Wang & Zhang, 2009), but in agreement with Sun, He, et al. (2009), we found that neutral polysaccharide had no antitumor activity, while the sulfated derivatives did. Hence, we speculate that the protein portion has a functional role in carbohydrate-induced inhibition. In contrast, sLNT exerted an inhibitory effect on free radicals, which can diffuse into leukemic leukocytes and generate iron-mediated oxidation-reduction reactions (Devi et al., 2000). As such, the sLNT generated using microwave radiation, which has an ordered, extended structure, exhibited enhanced antitumor activities.

## 4. Conclusions

Several studies have examined different polysaccharide fractions from the fruiting body of *L. edodes*, and their antitumor activities have been reported (Carbonero et al., 2008; Chihara et al., 1970; Fang et al., 2006; Ruan et al., 2005; Surenjav et al., 2006; Wang & Zhang, 2009), but the same has not been done for exopolysaccharides derived from the fermentation broth of *L. edodes*. The isolation of these polysaccharides from fermentation broth is more convenient and does not require harsh extraction steps, resulting in more homogenous polysaccharides with less deactivation of their bioactivities. Moreover, we demonstrated that microwave radiation of LNT under very mild conditions for repeated, short time spans reduces the sulfation time from 6 h to 6 min. In addition, sLNT produced by conventional heating or microwave radiation exhibited similar physicochemical properties, chemical structures and activities. It is important to note that microwave radiation may have additional effects on polysaccharides, which can make it an excellent auxiliary method for use in carbohydrate chemistry. The antiproliferative tumor effects of polysaccharides in vitro remain a highly disputed and unexplored topic. As our study has shown, sLNT derived from *L. edodes* exhibit strong antioxidant activities and several antitumor properties, and therefore, represent highly efficient, novel antitumor agents. As such, their further study is warranted.

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## References

Alban, S., & Franz, G. (2001). Partial synthetic glucan sulfates as potential new antithrombotics: A review. *Biomacromolecules*, *2*, 354–361.

Alcain, F. J., Buron, M. I., Rodriguezaguilera, J. C., Villalba, J. M., & Navas, P. (1990). Ascorbate free-radical stimulates the growth of a human promyelocytic leukemia-cell line. *Cancer Research*, *50*, 5887–5891.

Athukorala, Y., Kim, K. N., & Jeon, Y. J. (2006). Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. *Food and Chemical Toxicology*, *44*, 1065–1074.

Bao, X. F., Zhen, Y., Ruan, L., & Fang, J. N. (2002). Purification, characterization, and modification of T lymphocyte-stimulating polysaccharide from spores of *Ganoderma lucidum*. *Chemical and Pharmaceutical Bulletin*, *50*, 623–629.

Biemond, P., Swaak, A. J. G., & Koster, J. F. (1984). Protective factors against oxygen free-radicals and hydrogen-peroxide in rheumatoid-arthritis synovial-fluid. *Arthritis and Rheumatism*, *27*, 760–765.

Burcham, P. C. (1998). Genotoxic lipid peroxidation products: Their DNA damaging properties and role in formation of endogenous DNA adducts. *Mutagenesis*, *13*, 287–305.

Carbonero, E. R., Gracher, A. H. P., Komura, D. L., Marcon, R., Freitas, C. S., Baggio, C. H., et al. (2008). *Lentinus edodes* heterogalactan: Antinociceptive and anti-inflammatory effects. *Food Chemistry*, *111*, 531–537.

Chen, J. N., Wang, Y. T., & Wu, J. S. B. (2009). A glycoprotein extracted from golden oyster mushroom *Pleurotus citrinopileatus* exhibiting growth inhibitory effect against U937 leukemia cells. *Journal of Agricultural and Food Chemistry*, *57*, 6706–6711.

Chen, Y. Y., & Chang, H. M. (2004). Antiproliferative and differentiating effects of polysaccharide fraction from fu-ling (*Poria cocos*) on human leukemic U937 and HL-60 cells. *Food and Chemical Toxicology*, *42*, 759–769.

Chihara, G., Hamuro, J., Maeda, Y., Arai, Y., & Fukuoka, F. (1970). Fractionation and purification of polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk) Sing (an edible-mushroom). *Cancer Research*, *30*, 2776–2781.

Dace, R., McBride, E., Brooks, K., Gander, J., Buszko, M., & Doctor, V. M. (1997). Comparison of the anticoagulant action of sulfated and phosphorylated polysaccharides. *Thrombosis Research*, *87*, 113–121.

Dasgupta, N., & De, B. (2004). Antioxidant activity of *Piper betle* L. leaf extract in vitro. *Food Chemistry*, *88*, 219–224.

Devi, G. S., Prasad, M. H., Saraswathi, I., Raghu, D., Rao, D. N., & Reddy, P. P. (2000). Free radicals antioxidant enzymes and lipid peroxidation in different types of leukemias. *Clinica Chimica Acta*, *293*, 53–62.

Dinis, T. C. P., Madeira, V. M. C., & Almeida, L. M. (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid-peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry and Biophysics*, *315*, 161–169.

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

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Fang, N. B., Li, Q. L., Yu, S. G., Zhang, J. X., He, L., Ronis, M. J. J., et al. (2006). Inhibition of growth and induction of apoptosis in human cancer cell lines by an ethyl acetate fraction from shiitake mushrooms. *Journal of Alternative and Complementary Medicine*, *12*, 125–132.

Feng, Y. L., Li, W. Q., Wu, X. Q., Cheng, J. W., & Ma, S. Y. (2010). Statistical optimization of media for mycelial growth and exo-polysaccharide production by *Lentinus edodes* and a kinetic model study of two growth morphologies. *Biochemical Engineering Journal*, *49*, 104–112.

Gey, K. F. (1993). Prospects for the prevention of free-radical disease, regarding cancer and cardiovascular-disease. *British Medical Bulletin*, *49*, 679–699.

Ghosh, P., Adhikari, U., Ghosal, P. K., Pujol, C. A., Carlucci, M. J., Damonte, E. B., et al. (2004). In vitro anti-herpetic activity of sulfated polysaccharide fractions from *Caulerpa racemosa*. *Phytochemistry*, *65*, 3151–3157.

Guo, Z. H., Hu, Y. L., Wang, D. Y., Ma, X., Zhao, X. N., Zhao, B. K., et al. (2009). Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. *Vaccine*, *27*, 660–665.

Herbert, V., Shaw, S., Jayatilleke, E., & Stoplerkasdan, T. (1994). Most free-radical injury is iron-related - it is promoted by iron, hemin, holoferritin and vitamin-C, and inhibited by desferoxamine and apoferritin. *Stem Cells*, *12*, 289–303.

He, L., Yin, N., Cheng, J. W., Wu, X. Q., Jiang, J. X., & Song, X. L. (2009). Structural features of a new heteropolysaccharide from the fruit bodies of Melia azedarach and its effect on cytotoxic activity. *Fitoterapia*, *80*, 399–403.

Kelley, E. E., Buettner, G. R., & Burns, C. P. (1997). Production of lipid-derived free radicals in L1210 murine leukemia cells is an early oxidative event in the photodynamic action of photofrin(R). *Photochemistry and Photobiology*, *65*, 576–580.

Navarro, D. A., Flores, M. L., & Stortz, C. A. (2007). Microwave-assisted desulfation of sulfated polysaccharides. *Carbohydrate Polymers*, *69*, 742–747.

Ooi, V. E. C., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*, 715–729.

Otagiri, K., Ohkuma, T., Ikekawa, T., & Tanaka, S. (1983). Intensification of antitumor-immunity by protein-bound polysaccharide, EA6, derived from *Flammulina velutipes* (Curt. ex Fr.) Sing. combined with murine leukemia L1210 vaccine in animal experiments. *Journal of Pharmacobio-dynamics*, *6*, 96–104.

Papy-Garcia, D., Barbier-Chassefiere, V., Rouet, V., Kerros, M. E., Klochendler, C., Tournaire, M. C., et al. (2005). Nondegradative sulfation of polysaccharides. Synthesis and structure characterization of biologically active heparan sulfate mimetics. *Macromolecules*, *38*, 4647–4654.

Petit, E., Papy-Garcia, D., Muller, G., Courtois, B., Caruelle, J. P., & Courtois, J. (2004). Controlled sulfatation of natural anionic bacterial polysaccharides can yield agents with specific regenerating activity in vivo. *Biomacromolecules*, *5*, 445–452.

Pokhrel, C. P., & Ohga, S. (2007). Submerged culture conditions for mycelial yield and polysaccharides production by *Lyophyllum decastes*. *Food Chemistry*, *105*, 641–646.

Raghuraman, A., Riaz, M., Hindle, M., & Desai, U. R. (2007). Rapid and efficient microwave-assisted synthesis of highly sulfated organic scaffolds. *Tetrahedron Letters*, *48*, 6754–6758.

Reizenstein, P. (1991). Iron, free-radicals and cancer. *Medical Oncology and Tumor Pharmacotherapy*, *8*, 229–233.

Ruan, Z., Su, J., Dai, H. C., & Wu, M. C. (2005). Characterization and immunomodulating activities of polysaccharide from *Lentinus edodes*. *International Immunopharmacology*, *5*, 811–820.

Scherba, V. V., & Babitskaya, V. G. (2008). Polysaccharides of xylotrophic basidiomycetes. *Applied Biochemistry and Microbiology*, *44*, 78–83.

Singh, V., Tiwari, A., Tripathi, D. N., & Malviya, T. (2003). Microwave promoted methylation of plant polysaccharides. *Tetrahedron Letters*, *44*, 7295–7297.

Staub, A. M. (1965). Removal of protein—Sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Subrahmanyam, V. V., Ross, D., Eastmond, D. A., & Smith, M. T. (1991). Potential role of free-radicals in benzene-induced myelotoxicity and leukemia. *Free Radical Biology and Medicine*, *11*, 495–515.

Sun, Z. W., He, Y. L., Liang, Z. H., Zhou, W. W., & Niu, T. G. (2009). Sulfation of (1→3)-beta-D-glucan from the fruiting bodies of *Russula virescens* and antitumor activities of the modifiers. *Carbohydrate Polymers*, *77*, 628–633.

Sun, Y. X., Liang, H. T., Cai, G. Z., Guan, S. W., Tong, H. B., Yang, X. D., et al. (2009). Sulfated modification of the water-soluble polysaccharides from *Polyporus albicans* mycelia and its potential biological activities. *International Journal of Biological Macromolecules*, *44*, 14–17.

Surenjav, U., Zhang, L., Xu, X. J., Zhang, X. F., & Zeng, F. B. (2006). Effects of molecular structure on antitumor activities of (1→3)-beta-D-glucans from different *Lentinus edodes*. *Carbohydrate Polymers*, *63*, 97–104.

Suzuki, M., Kikuchi, T., Takatsuki, F., & Hamuro, J. (1994). Curative effects of combination therapy with lentinan and interleukin-2 against established murine tumors, and the role of Cd8-positive T-cells. *Cancer Immunology Immunotherapy*, *38*, 1–8.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry & Cell Biology*, *39*, 44–84.

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

Witvrouw, M., & DeClercq, E. (1997). Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *Vascular Pharmacology*, *29*, 497–511.

Wong, S. M., Wong, K. K., Chiu, L. C. M., & Cheung, P. C. K. (2007). Non-starch polysaccharides from different developmental stages of *Pleurotus tuber-regium* inhibited the growth of human acute promyelocytic leukemia HL-60 cells by cell-cycle arrest and/or apoptotic induction. *Carbohydrate Polymers*, *68*, 206–217.

Xing, R. E., Liu, S., Yu, H. H., Zhang, Q. B., Li, Z., & Li, P. C. (2004). Preparation of low-molecular-weight and high-sulfate-content chitosans under microwave radiation and their potential antioxidant activity in vitro. *Carbohydrate Research*, *339*, 2515–2519.

Xue, S. X., Chen, X. M., Lu, J. X., & Jin, L. Q. (2009). Protective effect of sulfated *Achyranthes bidentata* polysaccharides on streptozotocin-induced oxidative stress in rats. *Carbohydrate Polymers*, *75*, 415–419.

Yang, J. H., Du, Y. M., Wen, Y., Li, T. Y., & Hu, L. (2003). Sulfation of Chinese lacquer polysaccharides in different solvents. *Carbohydrate Polymers*, *52*, 397–403.

Yang, X. B., Gao, X. D., Han, F., & Tan, R. X. (2005). Sulfation of a polysaccharide produced by a marine filamentous fungus *Phoma herbarum* YS4108 alters its antioxidant properties in vitro. *Biochimica Et Biophysica Acta-General Subjects*, *1725*, 120–127.

Yoshida, T., Yasuda, Y., Mimura, T., Kaneko, Y., Nakashima, H., Yamamoto, N., et al. (1995). Synthesis of curdlan sulfates having inhibitory effects in-vitro against aids viruses Hiv-1 and Hiv-2. *Carbohydrate Research*, *276*, 425–436.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2006). Polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*, 4–19.

Zhang, L. N., Zhang, M., Zhou, Q., Chen, J. H., & Zeng, F. B. (2000). Solution properties of antitumor sulfated derivative of alpha-(1→3)-D-glucan from *Ganoderma lucidum*. *Bioscience Biotechnology and Biochemistry*, *64*, 2172–2178.

Zhang, Q. B., Li, N., Zhou, G. F., Lu, X. L., Xu, Z. H., & Li, Z. (2003). In vivo antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodophyta) in aging mice. *Pharmacological Research*, *48*, 151–155.

Zhao, X., Xue, C. H., Li, Z. J., Cai, Y. P., Liu, H. Y., & Qi, H. T. (2004). Antioxidant and hepatoprotective activities of low molecular weight sulfated polysaccharide from *Laminaria japonica*. *Journal of Applied Phycology*, *16*, 111–115.

Zhu, Y. Z., Huang, S. H., Tan, B. K. H., Sun, J., Whiteman, M., & Zhu, Y. C. (2004). Antioxidants in Chinese herbal medicines: A biochemical perspective. *Natural Product Reports*, *21*, 478–489.