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The Antioxidant Activities of $C_{3,6}$-Dibenzoylated Phenyl-Thiosemicarbazone-Chitosans

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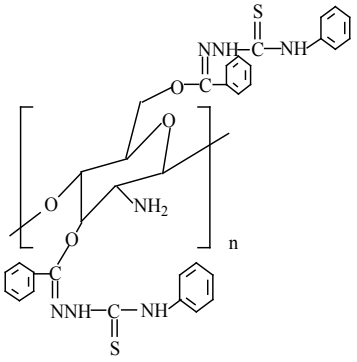
Abstract

Ten new $C_{3,6}$-dibenzoylated thiosemicarbazone-chitosan derivatives (with two different molecular weights) were prepared. The purpose of this study was to further investigate the antioxidant activities of this series of derivatives and the relationship between the structure and their antioxidant behavior. In order to know how the structure influenced the antioxidant activities of the derivatives, their structures were characterized by FT-IR spectroscopy and elemental analysis in this paper. In addition, the antioxidant activities of these new derivatives were also researched employing various established systems, such as hydroxyl radical (•OH)/superoxide anion (O_2^-)/DPPH scavenging and reducing power. Besides that, the cell toxicity of those derivatives was determined with MTT method. The results indicated that 3,6-DBZPTSCZLCS, 3,6-DBZ(*p*-NP)TSCZLCS, 3,6-DBZ(*o*-CP)TSCZHCS, and 3,6-DBZ(*p*-NP)TSCZHCS had the potential to be used as natural antioxidants because of their excellent activities in all these assays.



3,6-DBZPTSCZCS — 3,6-DBZPTSCZHCS , Mw= 200 KDa ;
3,6-DBZPTSCZLCS , Mw= 3 KDa .

Ten new 3, 6-diacetylated phenyl-thiosemicarbazone-chitosans were synthesized and characterized. The antioxidant activities of the derivatives were investigated.

Keywords: Chitosan; Located protection; Antioxidant activities; $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosans; Cell toxicity test

Introduction

Chitosan (CS), the linear (1-4)-2-amino-2-deoxy-β-D-glucan, randomly acetylated to a minor extent [1], is produced industrially from marine chitin [2]. As the only cationic polysaccharide in nature, CS has many satisfying features, including nontoxicity, biocompatibility and biodegradability.

Chemical modification of CS and its derivatives can enhance their properties and consequently expands their potential applications. And because of this, CS and its derivatives have been used in a number of different fields nowadays. They are an important class of N, S donor ligands which attract considerable interest because of their chemistry and biological activities, such as antitumor, antibacterial, antiviral, antiamoebic and antimalarial activities [3], so more and more people are researching their bioactivities. At present, there are all kinds of thiosemicarbazone derivatives, and their bioactivities were determined [4].

We have reported the preparation of acetyl and benzoyl phenyl-thiosemicarbazone derivatives of CS and their antimicrobial activities previously [5,6]. However; we have not prepared the derivatives with location method in all our previous reports. Thus, ten new $C_{3,6}$-dibenzoylated thiosemicarbazone derivatives of CS (with high and low molecular weight) were further prepared with located C_2-NH_2 protecting method under the existence of phthalic anhydride for the first time and their structures were characterized by FT-IR spectroscopy and elemental analysis in this essay. Then we assessed the antioxidant activities of this series of derivatives, and the relationship between their structures and antioxidant activities was studied in our present study. Besides that, the cell toxicity of those derivatives was determined with MTT method.

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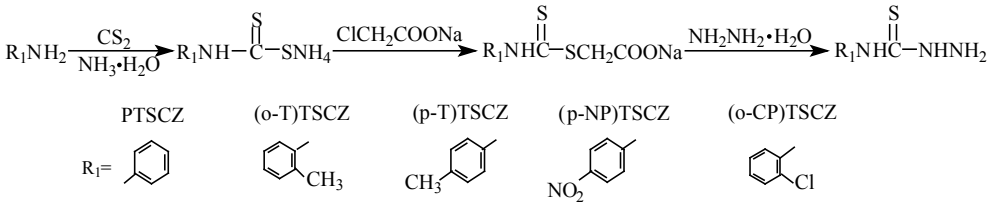
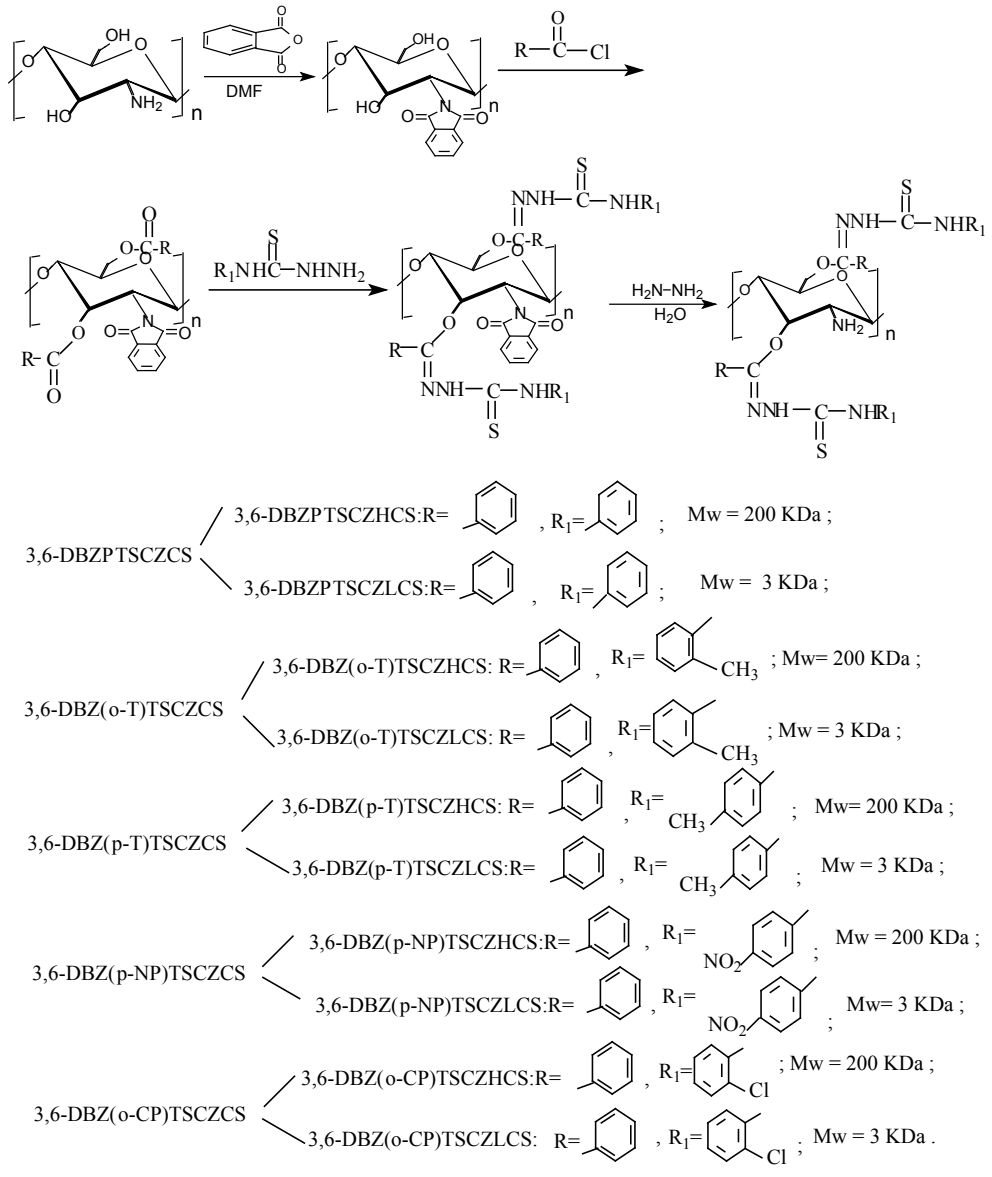
Methods

Chemistry

$C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosan derivatives were synthesized as shown in Schemes 1 and 2. Firstly, phenyl-thiosemicarbazones were prepared. Then 2-phenoxybenzyl dicarboxymido chitosan with two different molecular weights reacted with benzoyl chloride in trichloromethane and pyridine solution at 100℃ to give $C_{3,6}$-dibenzoylated chitosan. Finally, $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosans were synthesized by $C_{3,6}$-dibenzoylated chitosans reacting with phenyl-thiosemicarbazones. All of the products gave satisfactory spectroscopic data, which were in



Scheme 1: Synthesis pathway of phenyl-thiosemicarbazones.



Scheme 2: Synthesis pathway of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosans.

full accordance with their assigned structures. The antioxidant activities of the compounds were evaluated in an aqueous system *in vitro*.

Experimental

Materials

CS (deacetylation degree=95%) with high and low molecular weight (HCS/LCS) were supplied by Qingdao Yunzhou Biochemical Corp (China), average molecular weight 200 kDa and 3 kDa. Nitro blue tetrazolium (NBT), phenazine mothosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylene diamine tetra-acetic acid (EDTA), nicotinamide adenine dinucleotide reduced (NADH), trichloroacetic acid (TCA), potassium ferricyanide and ferric chloride were purchased from Sigma Chemicals Co., Ltd. (China). All other chemicals and reagents used in the preparation step were of analytical grade, and were used without further purification.

Analytical methods

Fourier transform infrared (FT-IR) spectra of the derivatives were measured in the 4,000-500 cm^{-1} regions using a Nicolet Nexus 670 FT-IR spectrometer. Elemental analysis (C, H, N, O, S) was performed on a Euro Analyzer EA3000 elemental analyzer. The viscometric average molecular weight of CS was estimated from the intrinsic viscosity determined in the solvent 0.1 M CH_3COOH/0.2 M NaCl using the Mark–Houwink parameter α=0.96, Kη=1.424 at 25°C. The intrinsic viscosity is expressed in mLg^{-1}.

The hydroxyl radical's scavenging ability of every sample was calculated as follows:

$$E\ (\%)=[(A_{sample}-A_{blank}) / A_{control}] \times 100$$

The superoxide radical's scavenging ability could be expressed like this:

$$E\ (\%)=(1-A_{sample} / A_{control}) \times 100$$

The reducing power was assessed with the absorbance value of each solution. The clearance toward DPPH free radical could be got from:

$$E\ (\%)=[1-(A_{sample}-A_{blank}) / A_{control}] \times 100$$

Preparation of phenyl-thiosemicarbazones

The Preparation of phenyl-thiosemicarbazones was operated as the method of Zhong [5]. 0.1 mol aniline (*o*-methylaniline, *p*-methylaniline, *o*-chloraniline or *p*-nitroaniline) was dissolved in 50 mL 95% ethanol. 20 mL ammonia and 8 mL carbon bisulfide was added drop wise when the temperature was under 30°C. After the mixture was placed for 2 h at room temperature, 0.1 mol sodium chloroacetate was added and the mixture was stirred for 30 min, then 12 mL hydrazine was added to the system. The mixture of products was concentrated to the half of the total volume, cooled to room temperature, filtered through a Buchner funnel under reduced pressure. The product was washed with water and dried at 55°C. A white powder was got after recrystallizing with 95% ethanol (The synthesis pathway of phenyl-thiosemicarbazones was shown below in Scheme 1).

Preparation of located benzoylated phenyl-thiosemicarbazone-chitosans

Preparation of 2-phenoxybenzyl dicarboximido chitosans: 2-phenoxybenzyl dicarboximido chitosans were prepared according to the method of Tao [7], 5 g CS (HCS/LCS) and 13.8 g phthalic anhydride were mixed well in 100 mL DMF solution, and then the mixture reacted at 90°C for 8 h until the mixture changed into a viscous transparent yellowish solution. Before pumping filtration, it was poured into ice water. Extracted it in a Soxhlet extractor with anhydrous alcohol for several hours, then it was dried with P_2O_5. The final product in this step was 2-phenoxybenzyl dicarboximido chitosan.

Preparation of protected $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosans: 3 g 2-phenoxybenzyl dicarboximido chitosan (with high/low molecular weight, HCS/LCS) was immersed in 60 mL trichloromethane and pyridine (1:1) for 24 h. 0.075 mol benzoyl chloride was added drop wise to the system under water-ice bath, then the mixture was stirred at 100°C for 12 h, cooled to room temperature and poured into a beaker containing 180 mL ethyl alcohol. After cooled at 4°C for 12 h, the mixture of products was filtered through a Buchner funnel under reduced pressure. The precipitate was rinsed with ethyl alcohol and dried at 55°C to get a powder precipitate. 0.5 mol benzoylated chitosan (3, 6-DBZHCS/3, 6-DBZLCS) was dissolved in 80 mL 2% acetic acid. 1 mol phenyl-thiosemicarbazone was poured into the system with stirring. After reacting 10 h at 100°C, the mixture was concentrated to 20 mL, cooled to the room temperature, and poured into a beaker containing 300 mL acetone. After cooled at 4°C for 12 h, the mixture of products was filtered through a Buchner funnel under reduced pressure. The precipitate was rinsed with acetone and dried at 55°C, and then a powder precipitate was produced.

Preparation of pure $C_{3,6}$-dibenzotylated phenyl-thiosemicarbazone-chitosans: There was a need of de-protection of phthalic anhydride protecting group in 2-phenoxybenzyl dicarboximido chitosan's benzoyl-thiosemicarbazone derivatives to get $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives. The method was as follows: add hydrazine hydrate into 2-phenoxybenzyl dicarboximido chitosan's benzoyl-thiosemicarbazone derivatives by molar ratio after fully dissolved them in water, react 3 h at 70°C, then concentrate. Repeat the steps above again, dialysis with distilled water to remove small ions, concentrate the volume to as little as possible, and then get solid by vacuum freeze-drying for 24 h. The product was $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone chitosan derivatives (The synthesis pathway of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone chitosan derivatives was shown below in Scheme 2).

Antioxidant Assays

Hydroxyl radical assays

The hydroxyl radical's scavenging ability of HCS/LCS and ten kinds of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosan (HCS/LCS) were assessed by the method of Halliwell. The scavenging ability was calculated as follows:

$$E\ (\%)=[(A_{sample}-A_{blank})/A_{control}] \times 100\ (1)$$

Where $A_{sample,}$ A_{blank} and $A_{control}$ were the absorbance of sample solution, blank solution, and the control solution respectively. So the final concentration of every sample was 25 μgmL^{-1}, 50 μgmL^{-1}, 100 μgmL^{-1}, 200 μgmL^{-1} and 400 μgmL^{-1}. Each experiment was performed three times, and the data were averaged.

Superoxide anion's scavenging assays

The superoxide radical's scavenging ability of HCS/LCS and ten kinds of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosan (HCS/LCS) were assessed by the method of Nishikimi [9]. The clearance could be expressed like this:

$$E\ (\%)=(1-A_{sample} / A_{control}) \times 100\ (2)$$

Where A_{sample} and $A_{control}$ were the absorbance of sample solutions and the control solution, respectively.

Each experiment was performed three times, and the data were averaged. So the final concentration of every sample was 25 μgmL^{-1}, 50 μgmL^{-1}, 100 μgmL^{-1}, 200 μgmL^{-1} and 400 μgmL^{-1}.

Reducing capacity assays

The reducing power of HCS/LCS and ten kinds of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosan (with high and low M_W) was quantified by the method described earlier by Yen [10]. Each experiment was performed three times, and the data were averaged. So the final concentration of every sample was 25 μgmL^{-1}, 50 μgmL^{-1}, 100 μgmL^{-1}, 200 μgmL^{-1} and 400 μgmL^{-1}.

DPPH free radical's scavenging assays

This part of assay was performed as the method of Curcio [11]. Take 35.49 mg DPPH, dissolve with 500 mL anhydrous ethanol and the concentration was 180 μmolL^{-1}. Dissolve every sample 0.0625 g with 25 mL anhydrous ethanol. Add 1.94, 1.88, 1.82, 1.76 and 1.52 mL secondary distilled water to 60, 120, 180, 240 and 480 μL sample solutions successively before adding 4.0 mL DPPH solution to the system, then shake with the plugs closed. Then place at room temperature for 20 min before determinating the absorbance data at 517 nm. The control of this assay was made up of 2.0 mL secondary distilled water and 4.0 mL DPPH solution. Replace 4.0 mL DPPH solution of the sample groups with 4.0 mL anhydrous ethanol to get the blank samples. The clearance could be got from:

$$E\ (\%)=[1-(A_{sample}-A_{blank}) / A_{control}] \times 100 \quad (3)$$

Where A_{sample}, A_{blank} and $A_{control}$ were the absorbance of sample solutions, blank solution and the control solution, respectively. Each experiment was performed three times, and the data were averaged. So, the final concentration of every sample was 25 μgmL^{-1}, 50 μgmL^{-1}, 75 μgmL^{-1}, 100 μgmL^{-1} and 200 μgmL^{-1}.

Results

Structure and physicochemical characteristics of benzoyl phenyl-thiosemicarbazone-chitosans elemental analysis results

Elemental analysis results of derivatives with high molecular weight: The results of elemental analysis, yield, and degree of linking of the high molecular weight derivatives were listed in Table 1. The yield of 3, 6-DBZPTSCZHCS, 3, 6-DBZ(*o*-T)TSCZHCS, 3, 6-DBZ(*p*-T) TSCZHCS, 3, 6-DBZ(*o*-CP)TSCZHCS and 3, 6-DBZ(*p*-NP)TSCZHCS was 69.2, 75.6, 75.1, 68.4 and 59.8%; respectively. Elemental analysis indicated that the degree of substitution of 3, 6-DBZPTSCZHCS, 3, 6-DBZ(*o*-T)TSCZHCS, 3, 6-DBZ(*p*-T)TSCZHCS, 3, 6-DBZ(*o*-CP) TSCZHCS and 3, 6-DBZ(*p*-NP)TSCZHCS was 42.6, 41.7, 45.9, 38.0 and 34.9% respectively.

Elemental analysis results of derivatives with low molecular weight: The results of elemental analysis, yield, and degree of linking of the low molecular weight derivatives were showed in Table 2. The yield of 3, 6-DBZPTSCZLCS, 3, 6-DBZ(*o*-T)TSCZLCS, 3, 6-DBZ(*p*-T) TSCZLCS, 3, 6-DBZ(*o*-CP)TSCZLCS and 3, 6-DBZ(*p*-NP)TSCZLCS was 58.0, 52.6, 49.9, 48.3 and 45.8%; respectively. Elemental analysis indicated that the degree of substitution of 3, 6-DBZPTSCZLCS, 3, 6-DBZ(*o*-T)TSCZLCS, 3, 6-DBZ(*p*-T)TSCZLCS, 3, 6-DBZ(*o*-CP) TSCZLCS and 3, 6-DBZ(*p*-NP)TSCZLCS was 32.1, 33.9, 32.6, 31.8 and 30.7% respectively.

FT-IR spectrum analysis of the derivatives with high molecular weight: Figure 1 showed the comparison of transmission FT-IR spectra for the derivatives with original CS (HCS) and located dibenzoylated-chitosan (3, 6-DBZHCS). As for HCS, the peak at 3447 cm^{-1} was caused by the stretching vibration of O-H and N-H, the peak appeared at 1596 cm^{-1} belonged to -NH_2. When it came to 3, 6-DBZHCS, the peak of the stretching vibration of O-H and N-H appeared at 3240 cm^{-1}, the peak of -NH_2 disappeared; the peaks appeared at 1710 cm^{-1} were due to C=O group. All of the information mentioned above indicated the preparation of C_2-NH_2 protected $C_{3,6}$-dibenzoylated chitosan. As for 3, 6-dibenzoylated phenyl-thiosemicarbazone-chitosan [3, 6-DBZPTSCZHCS], the sharp peaks appeared at 3310 cm^{-1} and 3130 cm^{-1} belonged to ν_{NH}, the peak of 1600 cm^{-1} was due to C=N group, the peaks at 1480 cm^{-1} and 1310 cm^{-1} were resulted from the overlapping of ν_{NH} and benzene, the peak of 1220 cm^{-1} belonged to C-N group, the peak appeared at 1285 cm^{-1} was because of C=S group, the peaks of benzene were at 1020 cm^{-1} and 754 cm^{-1}. According to the spectrum of 3, 6-dibenzoylated (*p*-tolyl)-thiosemicarbazone-chitosan [3, 6-DBZ(*p*-T)TSCZHCS], the sharp peaks appeared at 3420 cm^{-1} and 3120 cm^{-1} belonged to ν_{NH}, the peak of 1610 cm^{-1} was due to C=N group, the peaks at 1500 cm^{-1} and 1310 cm^{-1} were resulted from the overlapping of ν_{NH} and benzene, the peak of 1230 cm^{-1} belonged to C-N group, the peak appeared at 1312 cm^{-1} was because of C=S group, the peaks of benzene were at 880 cm^{-1} and 806 cm^{-1}. Compared to the spectrum of 3,6-DBZ(*p*-T)TSCZHCS, the spectra of 3, 6-dibenzoylated (*o*-tolyl)-thiosemicarbazone-chitosan [3, 6-DBZ(*o*-T)TSCZHCS] and 3, 6-dibenzoylated phenyl-thiosemicarbazone-chitosan [3, 6-DBZPTSCZHCS] were very similar to it. Compared with the spectrum of 3, 6-DA(*p*-T)TSCZHCS, there was a peak at 1650 cm^{-1} on the spectrogram of 3, 6-dibenzoylated (*p*-nitro-phenyl)-thiosemicarbazone-chitosan [3, 6-DBZ(*p*-NP)TSCZHCS], which belonged to -NO_2 group. For the same reason, 3, 6-dibenzoylated (*o*-chloride-phenyl)-thiosemicarbazone-chitosan [3, 6-DBZ (*o*-CP) TSCZHCS] was obtained. The results of low molecular weights' derivatives together with original chitosan (LCS) and located dibenzoyl-chitosan (3, 6-DBZLCS) were very similar to Figure 1, except that there were differences in the intensities among these peaks.

Compounds	Yield (%)	Elemental analysis (%)					Grafted degree (%)
		C	N	H	S	O	
HCS	–	48.12	7.27	6.96	–	20.25	–
3,6-DBZPTSCZHCS	69.2	52.68	14.33	4.02	8.87	16.03	42.6
3,6-DBZ(*o*-T)TSCZHCS	75.6	52.66	12.47	5.09	8.93	15.62	41.7
3,6-DBZ(*p*-T)TSCZHCS	75.1	53.11	13.58	4.26	9.20	16.09	45.9
3,6-DBZ(*o*-CP)TSCZHCS	68.4	52.86	15.28	4.61	7.66	14.50	38.0
3,6-DBZ(*p*-NP)TSCZHCS	59.8	49.21	14.75	4.06	7.52	15.83	34.9

Table 1: Elemental analysis results, yield and degree of grafted benzoyl phenyl-thiosemicarbazone derivatives of HCS.

Compounds	Yield (%)	Elemental analysis (%)					Grafted degree (%)
		C	N	H	S	O	
LCS	–	43.47	7.96	7.18	–	18.63	–
3,6-DBZPTSCZLCS	58.0	48.87	15.6	5.03	6.88	13.21	32.1
3,6-DBZ(*o*-T)TSCZLCS	52.6	51.09	14.25	5.2	7.27	14.05	33.9
3,6-DBZ(*p*-T)TSCZLCS	49.9	52.08	14.03	4.96	6.9	14.22	32.6
3,6-DBZ(*o*-CP)TSCZLCS	48.3	49.89	15.09	6.08	6.72	13.88	31.8
3,6-DBZ(*p*-NP)TSCZLCS	45.8	48.54	15.23	6.19	6.67	13.69	30.7

Table 2: Elemental analysis results, yield and degree of grafted benzoyl phenyl-thiosemicarbazone derivatives of LCS.

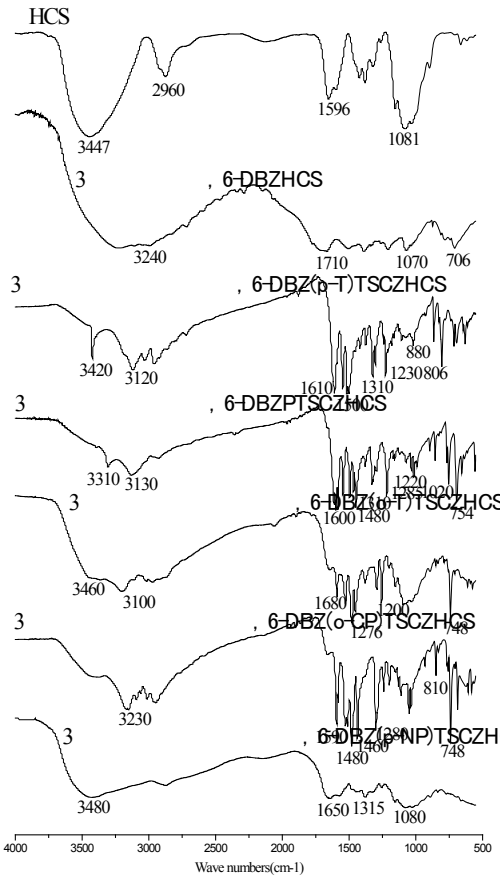


Figure 1: FT-IR spectrum data of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosans (HCS).

Discussion

Antioxidant activities

Hydroxyl free radical's scavenging abilities: Figures 2 and 3 showed the scavenging curves of V_C, HCS, LCS and their derivatives toward hydroxyl free radical. These curves indicated that all of the derivatives' (of both HCS and LCS) scavenging abilities were higher than the raw materials (HCS and LCS), and increased as the concentration increased. Moreover, those derivatives of LCS were much more active than the derivatives of HCS.

The max scavenging clearance and the min scavenging clearance of these derivatives of LCS were both superior to that of the HCS. Among those five derivatives of LCS, 3, 6-DBZ(*o*-CP)TSCZLCS had the best activity towards hydroxyl free radical, and the highest clearance was 76.70% when the concentration was 400 μgmL^{-1}. Among those five derivatives of HCS, 3, 6-DBZ(*o*-CP)TSCZHCS had the best activity toward hydroxyl free radical, and the highest clearance was 56.67% when the concentration was 400 μgmL^{-1}. All the derivatives of LCS and HCS had superior scavenging abilities than LCS and HCS. This result shows that the grafted phenyl-thiosemicarbazone group can increase the antioxidant activities of Chitosan. And the possible mechanism could be like this: hydroxyl free radical reacted with active hydrogen atoms in the derivatives and formed a most stable macromolecule radical. Because there were quantities of strong intermolecular and intramolecular hydrogen bonds in CS, its activity was not high. When the phenyl-thiosemicarbazone groups were grafted on Chitosan chain, the intermolecular and intramolecular hydrogen bonds were disrupted at the same time, so the antioxidant activities of the new derivatives were much higher than that of Chitosan.

On the other hand, molecular weight also affected the antioxidant activities of the derivatives, and the lower the molecular weight was the higher antioxidant activity the derivative had. This was accord with that of Xing [12], who indicated that lower molecular weight led to higher antioxidant activity. For example, though the grafted degree of 3, 6-DBZ(*p*-NP)TSCZLCS was 30.7%, but its best antioxidant activity reached 73.30%, which was much higher than that of 3, 6-DBZ(*p*-NP)TSCZHCS (38.99%), while the grafted degree of 3, 6-DBZ(*p*-NP) TSCZHCS was 34.9%.

In result, all the substituting groups improved the antioxidant activities of CS, and the order of the derivatives was that V_C>3,6-DBZ(*o*-CP)TSCZHCS>3,6-DBZ(*o*-T)TSCZHCS>3,6-DBZPTSCZHCS>3,6-DBZ(*p*-NP)TSCZHCS>3,6-DBZ(*p*-T)TSCZHCS>HCS; 3,6-DBZ(*o*-CP)TSCZLCS>V_C>3,6-DBZ(*p*-NP)TSCZLCS>3,6-DBZ(*p*-T) TSCZLCS>3,6-DBZPTSCZLCS>3,6-DBZ(*o*-T)TSCZLCS>LCS. Thus, 3,6-DBZ(*o*-CP)TSCZLCS would have the potential to be further studied for its higher scavenging activity than V_C.

Furthermore, the IC_{50} values of those lower molecular derivatives were 144.49 μgmL^{-1} (3, 6-DBZ(*o*-CP)TSCZLCS), 214.86 μgmL^{-1} (3, 6-DBZ(*p*-NP)TSCZLCS), 242.58 μgmL^{-1} (3, 6-DBZ(*p*-T) TSCZLCS), 494.61 μgmL^{-1} (3, 6-DBZ(*o*-T)TSCZLCS), 422.20 μgmL^{-1} (3, 6-DBZPTSCZLCS); while those of higher molecular derivatives were 162.73 μgmL^{-1} (3, 6-DBZ(*o*-CP)TSCZHCS), 333.44 μgmL^{-1} (3, 6-DBZ(*o*-T)TSCZHCS), 861.87 μg·mL^{-1} (3, 6-DBZ(*p*-T)TSCZHCS), 482.39 μg·mL^{-1} (3, 6-DBZPTSCZHCS) and 1577.54 μg·mL^{-1} (3, 6-DBZ(*p*-NP)TSCZHCS); respectively.

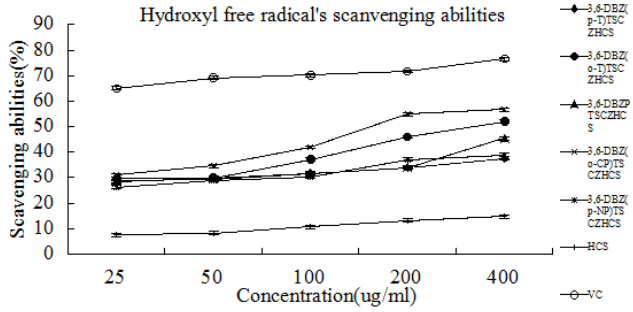


Figure 2: Scavenging curves of HCS, V_C, and derivatives towards hydroxyl free radical.

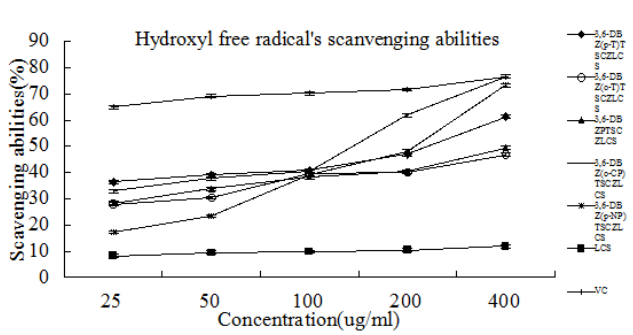


Figure 3: Scavenging curves of LCS, V_C, and derivatives towards hydroxyl free radical.

Superoxide anion's scavenging abilities: Figures 4 and 5 showed the scavenging curves of V_C, HCS, LCS and their derivatives toward superoxide anion. These curves indicated that all the derivatives (of both HCS and LCS) and the raw materials (HCS and LCS) showed strong scavenging abilities, and increased as the concentration increased. We deduced the mechanism was like this, the inner structure of Chitosan was severely disrupted by the introduction of grafted substituting groups after modification. The ability to form hydrogen bond declined sharply, and the hydroxyl and amino groups were activated, so this was helpful to the reaction with superoxide anion, because superoxide radical was a zwitterionic radical. It could react with free hydroxyl and amino groups in Chitosan and its derivatives.

Moreover, those derivatives of HCS were almost as active as the derivatives of LCS, which was not the same with that of Xing who indicated that CS derivatives with lower molecular weight had better scavenging abilities. All of the phenyl-thiosemicarbazones had similar strength in affecting the activities of those derivatives, despite the differences between molecular weights and grafted degrees. For example 3, 6-DBZ(*o*-T)TSCZHCS and 3, 6-DBZ(*p*-NP)TSCZLCS showed very similar activities in this assay, which two gained near maximum antioxidant values (90.15%, 90.83% respectively), though the grafted degree of these two was 41.7% and 30.7% respectively.

V_C and LCS had very similar scavenging abilities. And among those five derivatives of HCS, 3, 6-DBZ (*p*-NP) TSCZHCS had the best activity towards superoxide anion, and the highest clearance was 97.67% when the concentration was 400 μgmL^{-1}. Among those five derivatives of LCS, 3, 6-DBZ (*p*-T)TSCZLCS had the best activity towards superoxide anion and the highest clearance was 94.12% when the concentration was 400 μgmL^{-1}.

From these figures, we could know that the IC_{50} value of every sample was lower than 25 μgmL^{-1} except 3, 6-DBZ (*o*-CP) TSCZHCS (62.78 μgmL^{-1}). The order of the derivatives was that 3, 6-DBZ(*p*-NP)TSCZHCS>HCS>3,6-DBZ(*o*-CP)TSCZHCS>3,6-DBZ(*p*-T) TSCZHCS>V_C>3,6-DBZ(*o*-T)TSCZHCS>3,6-DBZPTSCZHCS; 3,6-DBZ(*p*-T)TSCZLCS>3,6-DBZ(*o*-CP)TSCZLCS>3,6-DBZPTSCZLCS>3,6-DBZ(*p*-NP)TSCZLC>V_C>LCS>3,6-DBZ(*o*-T) TSCZLCS.

Reducing abilities: Figures 6 and 7 showed the reducing curves of V_C, HCS, LCS and their derivatives. Reducing power was generally associated with the presence of reducing sugars because of their hydrogen-donating ability [14,15]. These curves indicated that the derivatives' (of both HCS and LCS) reducing abilities increased as the concentration increased. Moreover, those derivatives of LCS were much more active than the derivatives of HCS. That is to say, molecular weight also has an effect on the reducing power of these derivatives. And the lower molecular weight could lead to higher reducing ability. For example, when comparing the most active derivative of LCS and HCS, 3, 6-DBZ (*o*-CP) TSCZLCS (0.719) improved that of 3, 6-DBZ(*o*-T)TSCZHCS (0.373) by 92.76% when the concentration was 400 μgmL^{-1}. This was accord with that of Zhao [16], who showed that lower molecular weight led to higher activity.

Among those five derivatives of HCS, 3, 6-DBZ (*o*-T) TSCZHCS had the best reducing ability, and the highest index was 0.373 when the concentration was 400 μgmL^{-1}. Among those five derivatives of LCS, 3, 6-DBZ(*o*-CP)TSCZLCS had the best reducing ability, and the highest index was 0.719 when the concentration was 400 μgmL^{-1}. The order of the derivatives was that 3, 6-DBZ(*o*-CP)TSCZLCS>3,6-DBZ(*p*-NP)TSCZLCS>3,6-DBZ(*p*-T)TSCZLCS>3,6-DBZPTSCZLCS>3,6-DBZ(*o*-T)TSCZLCS>V_C>LCS; 3,6-DBZ(*o*-T)TSCZHCS>3,6-DBZ(*p*-NP)TSCZHCS>3,6-DBZ(*p*-T)TSCZHCS>V_C>3,6-DBZ(*o*-CP) TSCZHCS>3,6-DBZPTSCZHCS>HCS. That is to say, most of the derivatives prepared in this paper showed better reducing power than V_C. So this modification of thiosemicarbazones improved their reducing abilities to a high degree.

DPPH free radical's scavenging abilities: Figures 8 and 9 showed the scavenging curves of V_C, HCS, LCS and their derivatives toward DPPH free radical. These curves indicated that the derivatives' (of both HCS and LCS) scavenging abilities were increased as the concentration increased, and those derivatives of LCS all changed much more than those of HCS. 3, 6-DBZ(*p*-T)TSCZLCS improved its antioxidant activity by 58.59% in the assay range, 3, 6-DBZ(*o*-T)TSCZLCS improved by 52.02%, 3, 6-DBZPTSCZLCS improved by 69.69%, 3, 6-DBZ(*o*-CP) TSCZLCS improved by 12.12%, 3, 6-DBZ(*p*-NP)TSCZLCS improved by 51.52%; while the largest changing range of the five derivatives of HCS appeared at 3, 6-DBZPTSCZHCS, which had a 19.19% improving range.

Among those five derivatives of HCS, 3,6-DBZPTSCZHCS had the best activity towards DPPH free radical, and the highest clearance was 97.48% when the concentration was 200 μgmL^{-1}. Among those five derivatives of LCS, 3, 6-DBZPTSCZLCS had the best activity towards DPPH free radical, whose highest clearance was 98.99% when the concentration was 200 μgmL^{-1}. In this assay, the order of the derivatives was that 3,6-DBZPTSCZLCS>V_C>3,6-DBZ(*p*-T)TSCZLCS>LCS>3,6-DBZ(*o*-CP)TSCZLCS>3,6-DBZ(*o*-T)TSCZLCS>3,6-DBZ(*p*-NP) TSCZLCS; V_C>3,6-DBZPTSCZHCS>3,6-DBZ(*p*-T)TSCZHCS>3,6-DBZ(*o*-T)TSCZHCS>3,6-DBZ(*o*-CP)TSCZHCS>3,6-DBZ(*p*-NP) TSCZHCS>HCS. As the curves showed that most samples' IC_{50} value were lower than the minimum concentration used in this assay 25

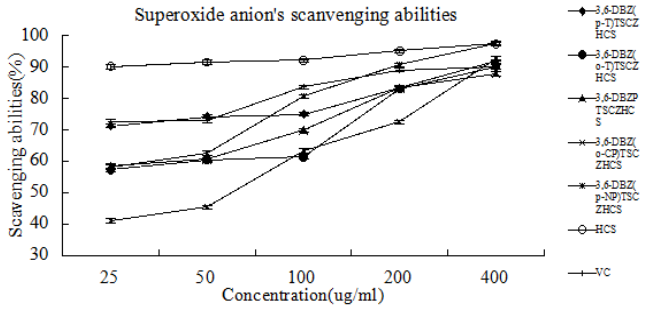


Figure 4: Scavenging curves of HCS, V_C, and derivatives towards superoxide anion.

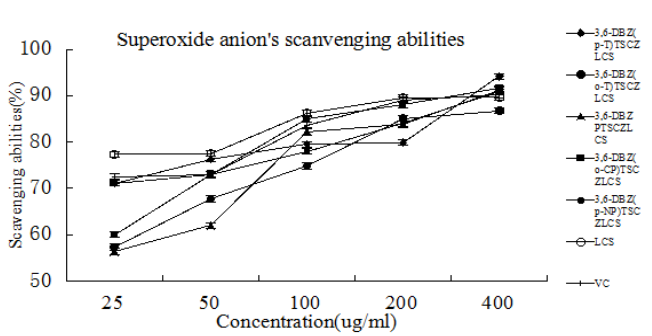


Figure 5: Scavenging curves of LCS, V_C, and derivatives towards superoxide anion.

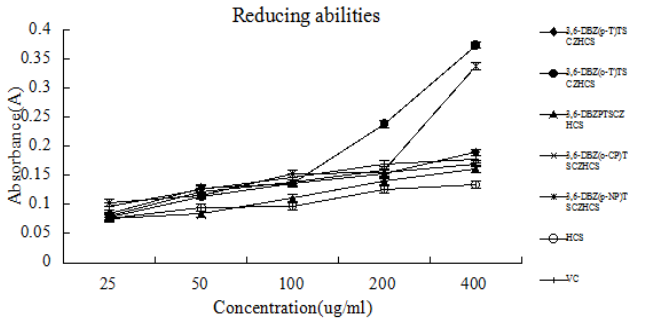


Figure 6: Curves of reducing abilities of HCS, V_C, and 5 kinds of derivatives.

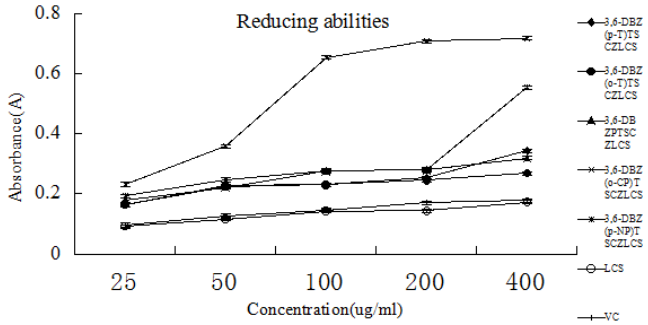


Figure 7: Curves of reducing abilities of LCS, V_C, and 5 kinds of derivatives.

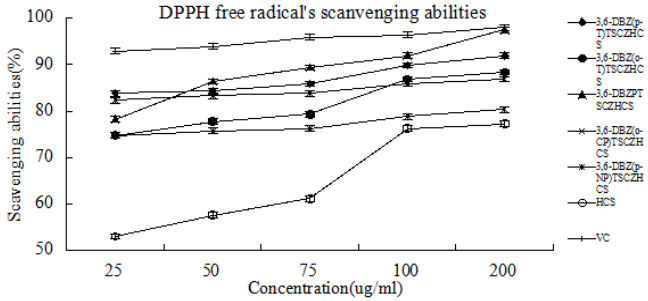


Figure 8: Scavenging curves of HCS, V_C, and 5 kinds of derivatives towards DPPH free radical.

μgmL^{-1}, except for 3, 6-DBZPTSCZLCS (39.86 μgmL^{-1}), 3, 6-DBZ(*p*-T)TSCZLCS (59.45 μgmL^{-1}) and 3, 6-DBZ(*p*-NP)TSCZLCS (74.93 μg·mL^{-1}).

In this test system, molecular weight also played an important role, and higher molecular weight showed higher activities. Those derivatives of HCS were much more active than the derivatives of LCS. Because the amount and the activity of hydroxyl and amino groups were important factors that associated with antioxidant activities of CS derivatives [16], the results were like these.

From this assay we could see the higher substituting degree of those derivatives of HCS displayed much acceptable activities compared with that of LCS. Hence, higher molecular weight higher substituting degree could gain better scavenging activities towards DPPH free radical. We thought this disagreement might be due to the rather different substituting groups we used in this experiment.

Tests for *in vitro* cytotoxicity: This part was operated according to the MTT method in MGC-803 cells with a Filter Max F5 Multi-mode Microplate Reader at 490 nm. Samples were all prepared into 2 mgmL^{-1} solutions and the final concentration gradient was 0.2 μL, 0.4 μL, 0.6 μL, 0.8 μL, 1.0 μL.

From the results of Figure 10 we could know only 3, 6-DBZPTSCZLCS and 3,6-DBZ(*p*-NP)TSCZLCS improved the cell viability of their corresponding thiosemicarbazones at the highest concentration. The other three derivatives of LCS (3,6-DBZ(*p*-T)TSCZLCS, 3,6-DBZ(*o*-T)TSCZLCS and 3,6-DBZ(*o*-CP)TSCZLCS) showed medium toxicity, but the differences were not so dramatic. The order of the cell viability of these five derivatives compared with original thiosemicarbazones and LCS was that 3,6-DBZ(*p*-NP)TSCZLCS>(*o*-CP)TSCZ>LCS>(*o*-T)TSCZ>3,6-DBZPTSCZLCS>3,6-DBZ(*o*-CP)TCZLCS>(*p*-NP)TSCZ>3,6-DBZ(*o*-T)TSZLCS>PTSCZ>(*p*-T)TSCZ>3,6-DBZ(*p*-T)TSCZLCS, so the toxicity order was just opposite. To draw a conclusion, 3, 6-DBZPTSCZLCS and 3,6-DBZ(*p*-NP)TSCZLCS might be used for some practical applications.

According to Figure 11, HCS had the best cell viability. Among the five derivatives of HCS, only 3,6-DBZ(*p*-T)TSCZHCS showed worse cell viability than (*p*-T)TSCZ; 3,6-DBZ(*o*-T)TSCZHCS, 3,6-DBZPTSCZHCS, 3,6-DBZ(*o*-CP)TSCZHCS and 3,6-DBZ(*p*-NP)TSCZHCS all had a better cell viability at the tested concentrations than their corresponding thiosemicarbazones. The order of the cell viability of these five derivatives compared with original thiosemicarbazones and HCS was that HCS>3,6-DBZPTSCZHCS>3,6-DBZ(*o*-T)TSCZHCS>3,6-DBZ(*p*-NP)TSCZHCS>(*o*-CP)TSCZ>3,6-DBZ(*o*-CP)TSCZHCS>(*o*-T)TSCZ>(*p*-NP)TSCZ>PTSCZ>(*p*-T)TSCZ>3,6-DBZ(*p*-T)TSCZHCS, so the toxicity order was just opposite. In other words, our modification decreased the toxicity of these four kinds of thiosemicarbazones ((*o*-T) TSCZ, PTSCZ, (*o*-CP) TSCZ and (*p*-NP) TSCZ), and these compounds could be researched further for applications.

Summary

Ten new different 3, 6-dibenzoylated phenyl-thiosemicarbazone derivatives of chitosans were synthesized in this paper. The antioxidant activities of HCS/LCS and their derivatives were investigated from four aspects. The results indicated that the increased antioxidant activities of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-chitosans might be attributed to benzoyl phenyl-thiosemicarbazone group that was grafted onto chitosans. And the results also demonstrated that the antioxidant activities of them were affected by their molecular weight together with grafted degree obviously. Lower molecular weight resulted in better antioxidant activities in the scavenging abilities toward hydroxyl free radical, superoxide anion and reducing abilities' assays. On the other hand, higher molecular weight showed better results in DPPH free radical's scavenging abilities. This was because these derivatives of

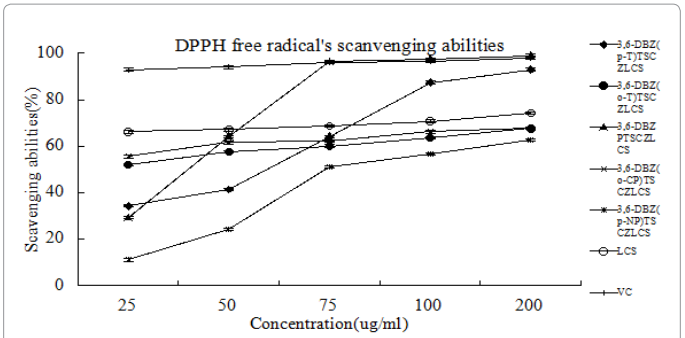


Figure 9: Scavenging curves of LCS, V_C, and 5 kinds of derivatives towards DPPH free radical.

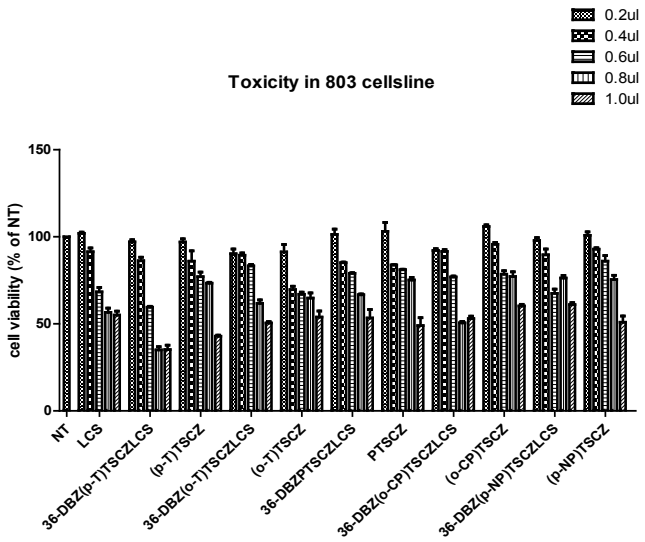


Figure 10: Toxicity of 5 kinds of derivatives of LCS in MGC-803 cells.

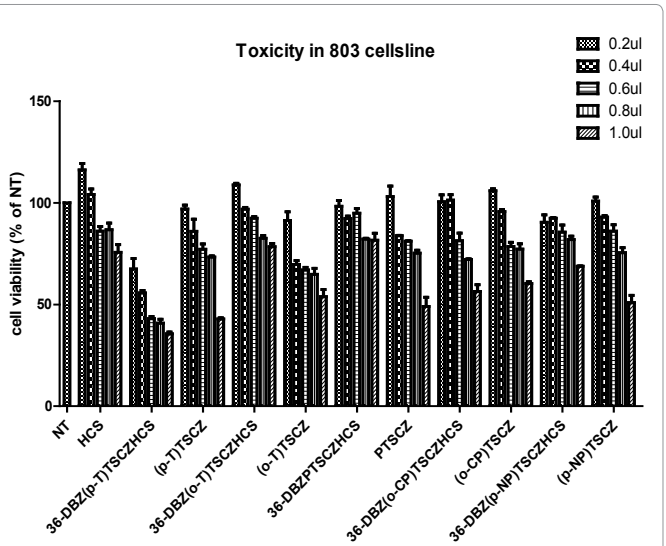


Figure 11: Toxicity of 5 kinds of derivatives of HCS in MGC-803 cells.

HCS were better substituted, and this was accord with our previous assumption that higher grafted degree could result in a stronger antifungal activity. These phenomena were accord with the reacting mechanism. To draw a conclusion, 3,6-DBZPTSCZLCS, 3,6-DBZ(*p*-NP)TSCZLCS, 3,6-DBZ(*o*-CP)TSCZHCS and 3,6-DBZ(*p*-NP) TSCZHCS had the potential to be used as natural antioxidants because of their low toxicity and high antioxidant activities. But there was still much work needing to be done, such as improving the water-soluble capacities and cell viability of the derivatives. In our future work, we would make efforts toward these directions.

Acknowledgements

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