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Study on the Antimicrobial Activity of $C_{3,6}$- Dibenzoylated Phenyl-Thiosemicarbazone-Chitosan Derivatives

Liu Yang and Zhimei Zhong*

College of Science, Inner Mongolia Agricultural University, Hohhot 010018, China

Abstract

The inhibitory effects of ten $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone- chitosan derivatives on four species of animal pathogenic bacteria and four kinds of plant pathogenic fungi were studied. The antimicrobial activity of all derivatives is superior to that of raw chitosan. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the derivatives against *Escherichia coli* were 7.40 μg·mL^{-1} and 14.80 μg·mL^{-1} respectively. The bacteriostatic effect of derivative on Gram-negative bacteria is stronger. All of the derivatives had a certain antifungal effect on the tested fungi in the concentration range of 0.05 mg·mL^{-1}-2 mg·mL^{-1}, with a maximum inhibition index of 100%. The antimicrobial action of the derivatives increased with the increase of concentration.

Keywords: Antibacterial activity; Antifungal activity; Chitosan; Derivatives; Dibenzoylated phenyl-thiosemicarbazone

Introduction

Chitosan is the only alkaline polysaccharide in nature formed by the deacetylation of chitin, which is an inexhaustible biological resource for human beings [1]. Chitosan has many excellent biological, chemical and physical properties, such as biodegradation, biocompatibility, antibacterial activity, non-toxic and can occur many kinds of chemical reactions [2]. Because of this, Chitosan has been widely used in many fields, such as cosmetics, food packaging, agriculture, environmental protection and so on [3]. But Chitosan is insoluble in water, alkaline solution, sulfuric acid and phosphoric acid, which greatly limits its application [4]. Based on the above characteristics, more and more experts and scholars began to study chitosan derivatives as antibacterial drugs, with the aim of obtaining new pesticides with good solubility, low toxicity and little environmental pollution. Vo and Lee [5] have been prepared hydrophobically modified Chitosan (HMCS) by Schiff base reaction and proved its potential as a green and sustainable antibacterial coating material by its antibacterial properties against *Escherichia coli*. Wang et al. [6] synthesized Chitosan (SP-CS) with various degrees of substitution (DS) and tested their antibacterial activities. The results showed that the antimicrobial activity of SP-CS enhanced by the introduction of sulfopropyl and increased with increasing DS. Ref. [7] tested Chitosan (CS) and its derivative Chitosan oligosaccharide lactate (COL) against *Aeromonas hydrophila*, *Edwardsiella ictaluri* and *Flavobacterium columnare* three highly pathogenic bacteria. The findings suggest that Chitosan is a promising alternative antibiotic as an antimicrobial agent to reduce disease outbreaks in aquaculture fish. Therefore, the research and development of chitosan antibacterial drugs has become the focus of research in recent years, the research and development of chitosan antibacterial drugs has become a hotspot in recent years.

Thiosemicarbazone derivatives have a wide range of biological activities, such as antibacterial, antiviral and antitumor [8]. Kulandaivelu et al. [9] synthesized thiosemicarbazones of p-aminobenzoic acid (PABA) and their antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Vibrio cholera* and *Bacillus subtilis* were tested. The experimental results show the derivatives have better antimicrobial and anticancer activity than their acid counterpart. Marina Azevêdo Souza et al. [10] prepared thiosemicarbazone and semicarbazone derivatives of lapachol. The minimal inhibitory concentrations (MIC) of thiosemicarbazone and semicarbazone derivatives of lapachol on *Enterococcus faecalis* and *Staphylococcus aureus* is 0.05 and 0.10 mol·mL^{-1}, respectively. Although thiosemicarbazone has good antibacterial activity, it has great toxicity, which limits its application to a great extent.

In summary, chitosan thiosemicarbazone compounds can not only reduce the toxicity of thiourea, but also improve the solubility of chitosan, and the cost is low. This research group synthesized, for the first time, $C_{3,6}$-dibenzoylated phenyl- thiosemicarbazone derivatives of Chitosan [11]. In this paper, the antimicrobial activity of these ten new derivatives against four species of animal pathogenic bacteria and four kinds of plant pathogenic fungi. The antimicrobial activity of the compounds was compared with that of Chitosan, thiosemicarbazone crystal, positive contrast drug and phenyl-thiosemicarbazone-chitosan without location protection. And the mechanism of bacteriostasis was discussed preliminarily. We hope that we can find several kinds of precursor compounds with good bacteriostatic effect, which will provide theoretical basis for the research and development of new pesticides and veterinary drugs.

Experimental

Materials

Chitosan (CS) was supplied by Qingdao Yunzhou Biochemistry Co. Ltd. (Qingdao, China, No: E3E56, respectively), with average molecular weight of 200 kDa and 3 kDa. Its deacetylation was 96%. *Alternaria solani* (BNCC227616), *F. oxysporum f. sp. cucumerinum* (BNCC 227992), *C. gloeosporioides (Penz.) Saec* (BNCC 114936) and *F. oxysporum f. sp. vasinfectum* (BNCC226606), *Escherichia coli*

***Corresponding author:** Zhimei Zhong, College of Science, Inner Mongolia Agricultural University, Hohhot 010018, China, Tel: +8618104709001; Fax: +864714309247; E-mail: zhimeihappy@126.com

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(BNCC 336902), *Micrococcus luteus* (BNCC 102589) was purchased from BeNa Cultuae Collection. *Staphylococcus aureus* (FSCC223001) and *Pseudomonas aeruginosa* (FSCC 206003) was purchased from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd.

Antibacterial assays

In the 96-well microtiter plates with a single pore volume of 400 μL, the drug was diluted by stepwise dilution method to obtain twelve concentrations (3789.6, 1894.8, 947.4, 473.7, 236.8, 118.4, 59.21, 29.61, 14.80, 7.40, 3.70, 1.85 $\mu g \cdot mL^{-1}$) sample solutions. Then add 20 μL bacteria liquid to each hole. With Norfloxacin as a positive control, the selection of a group without sample as blank control, each drug to do three groups of parallel experiments Put the culture plate in an incubator of 37°C for 24-48 h. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined by OD value. The OD value of the sample was determined by the enzyme analyzer and the bacteriostatic rate was calculated as follows:

$$\text{Antimicrobial rate } (\%)=(OD_b-OD_a)/OD_b$$

OD_a is the OD value of the sample, OD_b is the OD value of the blank control.

The concentration of antimicrobial rate exceeding 50% corresponds to the MIC. The concentration of antimicrobial rate exceeding 99.9% corresponds to the MBC.

Antifungal assays

The antifungal properties of fungi were performed based on the method of Zhong et al. [12]. The drug was mixed with the medium and prepared into a medium with a concentration of 0.05 $mg \cdot mL^{-1}$, 0.1 $mg \cdot mL^{-1}$, 0.5 $mg \cdot mL^{-1}$, 1 $mg \cdot mL^{-1}$ and 2 $mg \cdot mL^{-1}$. Then two funguses with a diameter of 4 mm were inoculated on each medium. After 48-72 h in the incubator of 29°C, the diameter of colony was measured and the bacteriostasis rate was calculated. The experiment was conducted with the same concentration of Wuyi and Haopu oligosaccharides as positive control and distilled water as negative control. Each drug did three parallel experiments. The inhibitory index was calculated as follows:

$$\text{Inhibitory index } (\%)=(D_b-D_a)/(D_b-4) \times 100$$

D_a is the diameter of the growth zone in the test plate and D_b is the diameter of growth zone in the control plate.

Results and Discussion

Antibacterial activity of the derivatives against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Micrococcus luteus*

The results show, that all the $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan had antibacterial activity to the test strains, and the bacteriostatic effect was better than the unmodified chitosan. The minimum value of MIC and MBC of the derivatives against *Escherichia coli* was 7.40 $\mu g \cdot mL^{-1}$ and 14.80 $\mu g \cdot mL^{-1}$ respectively. Antimicrobial activity of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against Gram-negative bacteria is better than gram-positive bacteria. Because *Pseudomonas aeruginosa* and *Micrococcus luteus* have strong drug resistance, the antibacterial effect of *Escherichia coli* and *Staphylococcus aureus* is better than that of *Pseudomonas aeruginosa* and *Micrococcus luteus*. The reasons for this result are related to the inhibition mechanism of different molecular weight chitosan, the structure of bacteria and the substitution degree of thiosemicarbazone. The cell walls of gram negative bacteria have two layers, which are divided into inner wall and outer wall. The inner wall layer is close to the cell membrane and consists of only peptidoglycan molecules. The outer wall layer is located outside the peptidoglycan layer and consists of lipopolysaccharide, phospholipids and protein layers. Protein and phosphoric acid are negatively charged, and if chitosan is protonated, electrostatic bonding occurs between the two. Because of the combination of the positive charge of the chitosan and the negatively charged substance in the cell, the essential amino acid solution is blocked from entering the cell, which also hinders the transport of proteins and other substances, leading to the death of the bacterium [13]. The lower the molecular weight of chitosan, the easier it is to enter the cell wall structure and bind with negatively charged substances to interfere with cell metabolism. Therefore, low molecular weight chitosan inhibited the gram negative bacteria more strongly. The cell walls of gram positive bacteria are monolayer, mainly consisting of peptidoglycan. The main antimicrobial mechanism of chitosan against gram positive bacteria is based on the formation of polymer chitosan polymer membranes that prevent nutrients from entering the cell or leaking [14]. Therefore, high molecular weight chitosan could be more effective against gram positive bacteria. As can be seen from Figures 1-4 and Table 1, the thiosemicarbazone crystal has a strong antibacterial activity, so the degree of substitution of thiosemicarbazone has a direct impact on the antibacterial effect. The better the antibacterial effect of the thiosemicarbazone crystal, the higher the substitution degree, the better the derivative antibacterial activity. Therefore, the bacteriostatic effect of the derivative is not completely followed by the high and low molecular weight chitosan on the bacteria inhibition rules.

Antifungal activities of the derivatives against *Alternaria solani, F. oxysporum f. sp. vasinfectum, C. gloeosporioides (Penz.) Saec.* and *F. oxysporum f. sp. cucumerinum*

Fungal diseases cause great losses to agricultural production every year. *Alternaria solani* is one of the important diseases of tomato, it can cause deciduous, fruit drop and broken branches, which has great effect on yield, and can caused cuts or even [15,16]. *F. oxysporum f. sp. cucumerinum* is an important disease in cucumber production, which causes serious losses to cucumber production. *Botrytis cinerea* has the characteristics of high reproduction rate, genetic variability and suitability. Over the years, a large number of chemicals have been used to make bacteria resistant to certain fungicides [17]. *F. oxysporum f. sp. vasinfectum* is one of the pathogens which have systemic infection, soil dissemination, stress resistance and pathogenicity. This germ is extremely difficult to control [18]. The *C. gloeosporioides (Penz.) Saec* is also called late rot disease and bitter rot disease, which is one of the most important diseases that endanger the fruit of grape [19]. Traditional pesticides have great environmental pollution and toxicity, so it is necessary to develop environmentally friendly and low toxic pesticides.

It is shown from Figures 5-8 that $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan have antifungal effect on four kinds of pathogenic fungi, and the antifungal effect is better than that of Chitosan. The inhibitory rate of some derivatives in certain concentration range was even higher than that of positive control. The antifungal effects of different derivatives on different strains were not the same. The antifungal effect of 3,6-DBAPTSCZHCS, 3,6-DBA(o-T) TSCZHCS, 3,6-DBA(p-T)TSCZHCS and 3,6-DBA(p-NP)TSCZHCS on *Alternaria solani* was very good, the inhibition rate reached 83.33%, 85.19%, 83.33%, 85.19%, respectively. The antifungal effect was more than that of the positive control. The effect of 3,6-DBAPTSCZLCS,

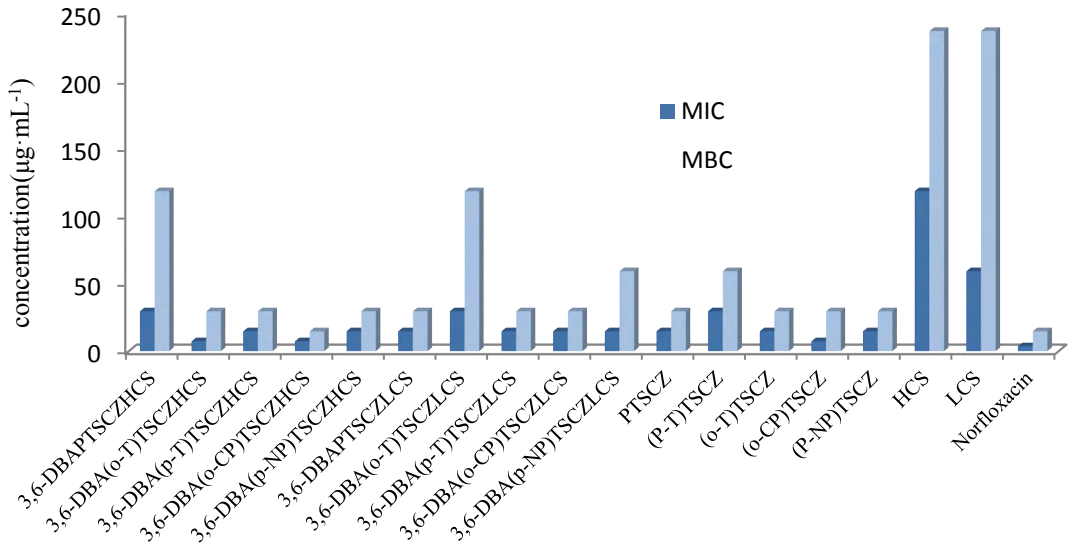

Figure 1: The MIC and MBC of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *Escherichia coli*.

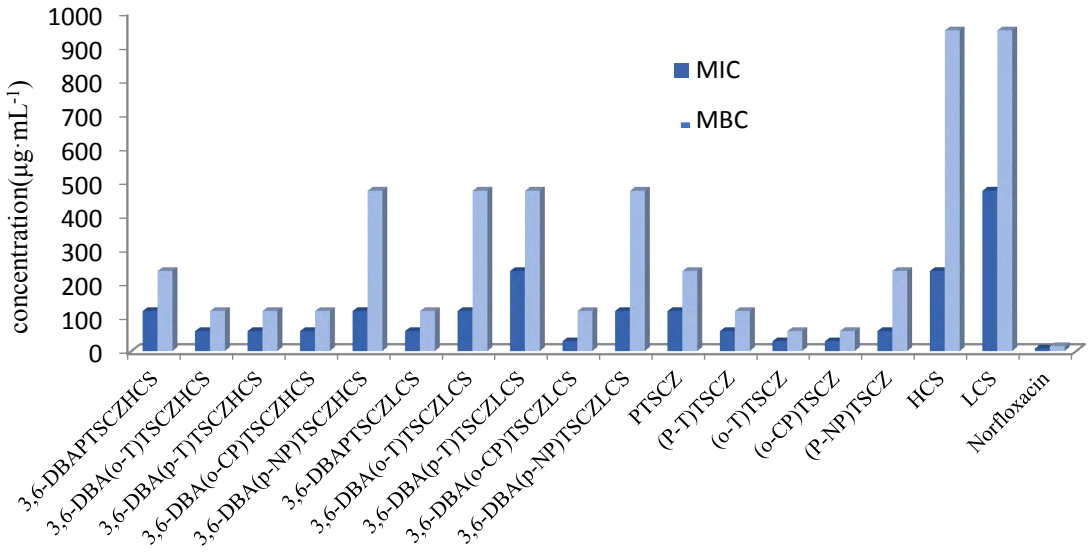

Figure 2: The MIC and MBC of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *Staphylococcus aureus*.

Sample	MIC, MBC (μg·mL^{-1})			
	P. aeruginosa	***M. luteus***	***E. coli***	***S. aureus***
HCS	947.4, 1894.8	947.4, 1894.8	118.4, 236.8	236.8,947.4
LCS	473.3, 1894.8	947.4, –a	59.21, 236.8	473.7,947.4
Norfloxacin	7.40, 14.80	14.80, 29.61	3.70, 14.80	7.40, 14.80
PTSCZ	118.4, 236.8	236.8, 473.7	14.80, 29.61	118.4, 236.8
(P-T)TSCZ	118.4, 236.8	118.4, 236.8	29.61, 59.21	59.21, 118.4
(o-T)TSCZ	59.21, 118.4	118.4, 236.8	14.80, 29.61	29.61, 59.21
(o-CP)TSCZ	29.61, 118.4	59.21, 118.4	7.40, 29.61	29.61, 59.21
(P-NP)TSCZ	118.4, 236.8	118.4, 473.7	14.80, 29.61	59.21, 236.8
3,6-DBAPTSCZHCS	236.8, 473.7	473.7, 947.4	29.61, 118.4	118.4, 236.8
3,6-DBA(o-T)TSCZHCS	236.8, 473.7	236.8, 947.4	7.40, 29.61	59.21, 118.4
3,6-DBA(p-T)TSCZHCS	59.21, 473.7	236.8, 947.4	14.80,29.61	59.21, 118.4

3,6-DBA(o-CP)TSCZHCS	118.4, 473.7	236.8, 473.7	7.40, 14.80	59.21, 118.4
3,6-DBA(p-NP)TSCZHCS	473.3, 947.4	473.7, 947.4	14.80, 29.61	118.4, 473.7
3,6-DBAPTSCZLCS	236.8, 473.7	473.7, 947.4	14.80, 29.61	59.21, 118.4
3,6-DBA(o-T)TSCZLCS	118.4, 236.8	236.8, 473.7	29.61, 118.4	118.4, 473.7
3,6-DBA(p-T)TSCZLCS	236.8, 473.7	236.8, 947.4	14.80, 29.61	236.8, 473.7
3,6-DBA(o-CP)TSCZLCS	118.4, 473.7	236.8, 473.7	14.80, 29.61	29.61, 118.4
3,6-DBA(p-NP)TSCZLCS	236.8, 473.7	236.8, 947.4	14.80, 59.21	118.4, 473.7

[a]Not determined under the test condition

Table 1: MIC and MBC values of the samples against *Eschercichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Microccus luteus.*

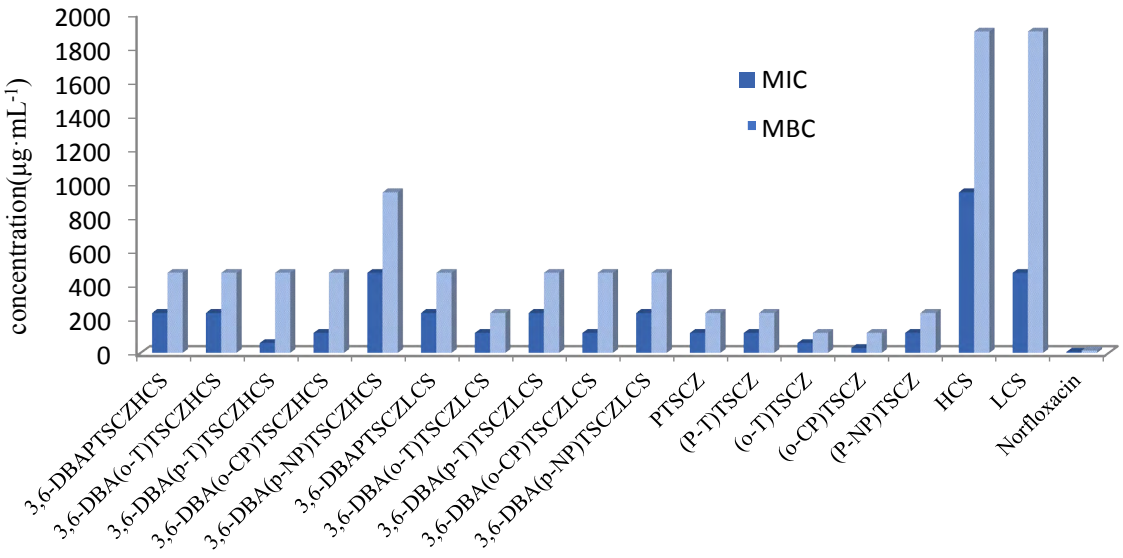

Figure 3: The MIC and MBC of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *Pseudomonas aeruginosa.*

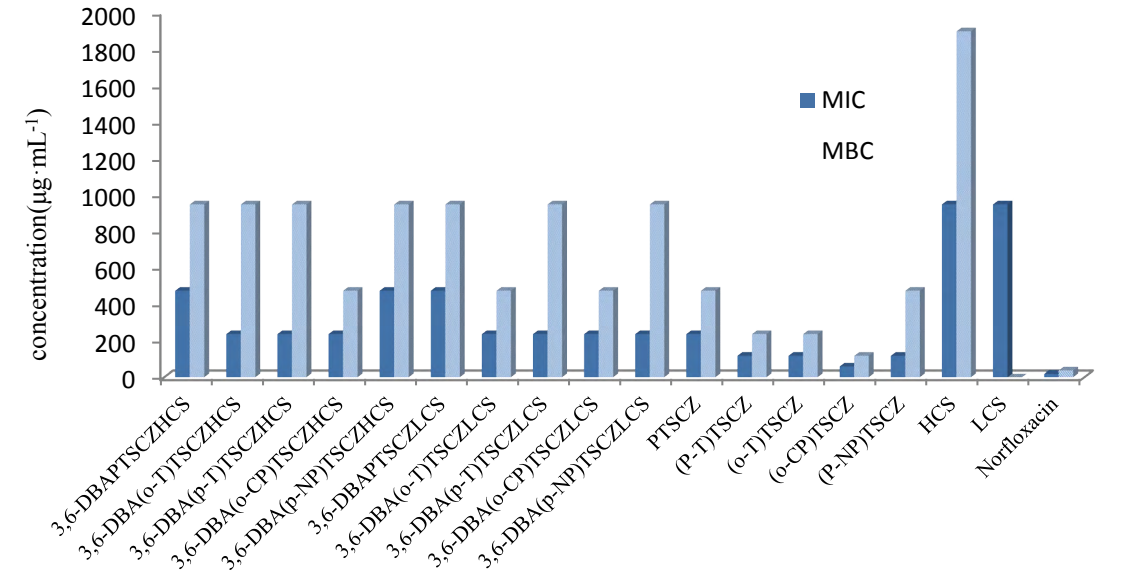

Figure 4: The MIC and MBC of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *Microccus luteus.*

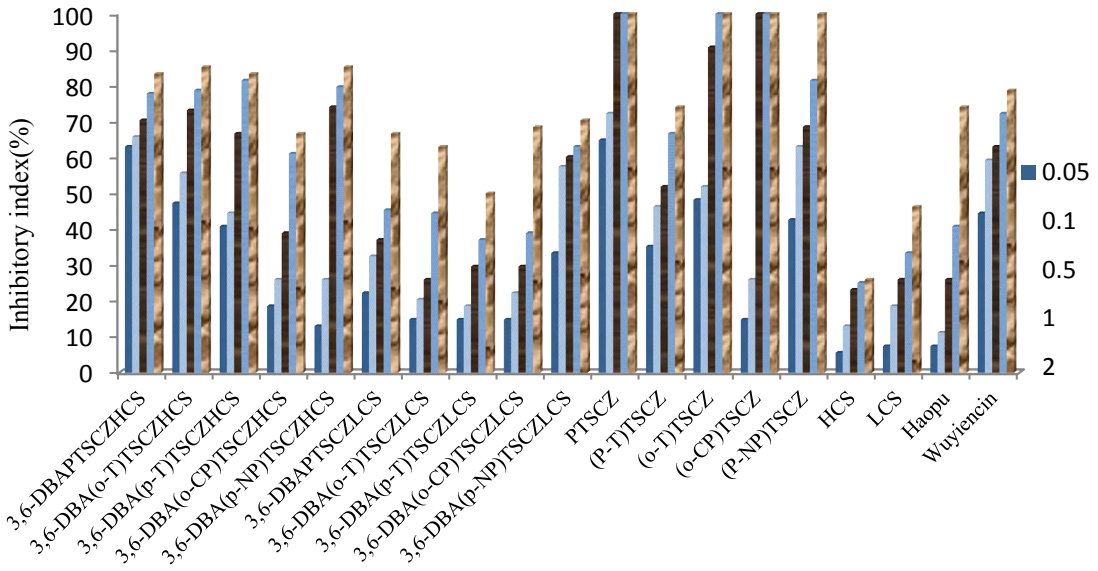

Figure 5: Antifungal activities of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *Alternaria solani*.

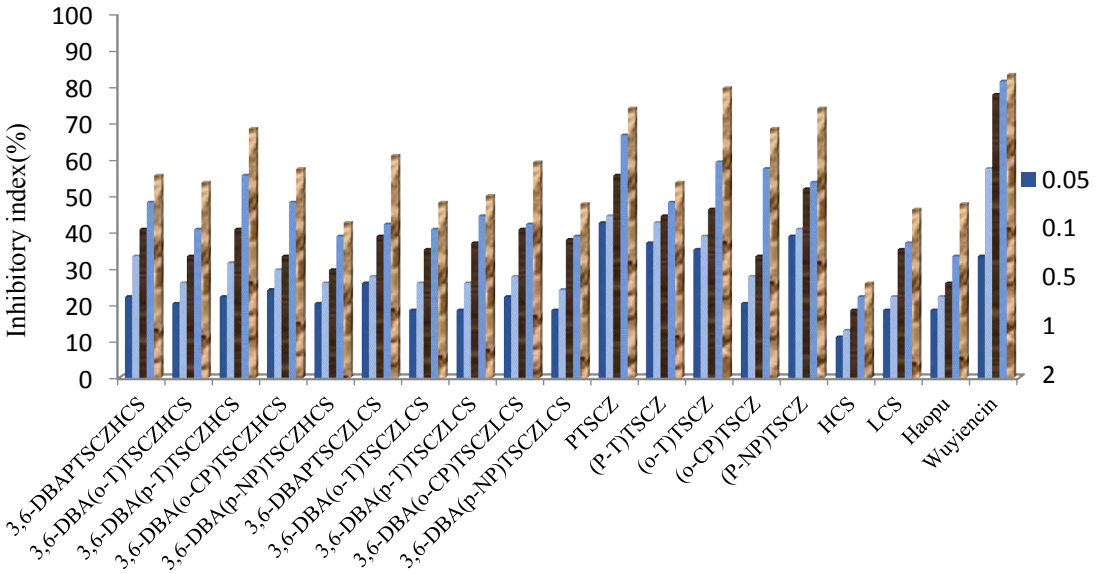

Figure 6: Antifungal activities of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *F. oxysporum f. sp. cucumerinum*.

3,6-DBA(o-CP)TSCZLCS and 3,6-DBA(p-NP)TSCZLCS on the control of *Alternaria solani* was also good, and the inhibition rates were reached 66.67%, 68.52%, 74.07%, respectively. 3,6-DBAPTSCZHCS, 3,6-DBA(o-T)TSCZHCS, 3,6-DBA(p-T)TSCZHCS, 3,6-DBA(o-CP)TSCZHCS, 3,6-DBAPTSCZLCS and 3,6-DBA(o-CP)TSCZLCS has good antifungal effect on *F. oxysporum f. sp. cucumerinum*, and the inhibitory rates were 55.56%, 53.7%, 68.52%, 57.41%, 61.11% and 59.26%, respectively. The antifungal effect was better than that of the positive control (Haopu). For *F. oxysporum f. sp. vasinfectum*, penicillin, the antifungal rate of 3,6-DBAPTSCZHCS, 3,6-DBA(o-T) TSCZHCS and 3,6-DBA(p-T)TSCZHCS reached 100%. 3,6-DBA(o-CP)TSCZHCS, 3,6-DBA(p-NP)TSCZHCS and 3,6-DBAPTSCZLCS also have a better inhibitory effect, and the inhibitory rates were 81.82%, 81.82%, 90.91%, respectively. Antifungal effect was stronger than two positive control drugs. The inhibitory rate of 3,6-DBA(o-CP) TSCZLCS on *F. oxysporum f. sp. vasinfectum* was 77.27%, which is better than that of positive control (Haopu). For the *C. gloeosporioides (Penz.) Saec*, 3,6-DBA(o-T)TSCZHCS, 3,6-DBA(p-T)TSCZHCS, 3,6-DBA(o-CP)TSCZHCS, 3,6-DBAPTSCZLCS and 3,6-DBA(o-CP) TSCZLCS had better antifungal effect, and the rate of fungi inhibition

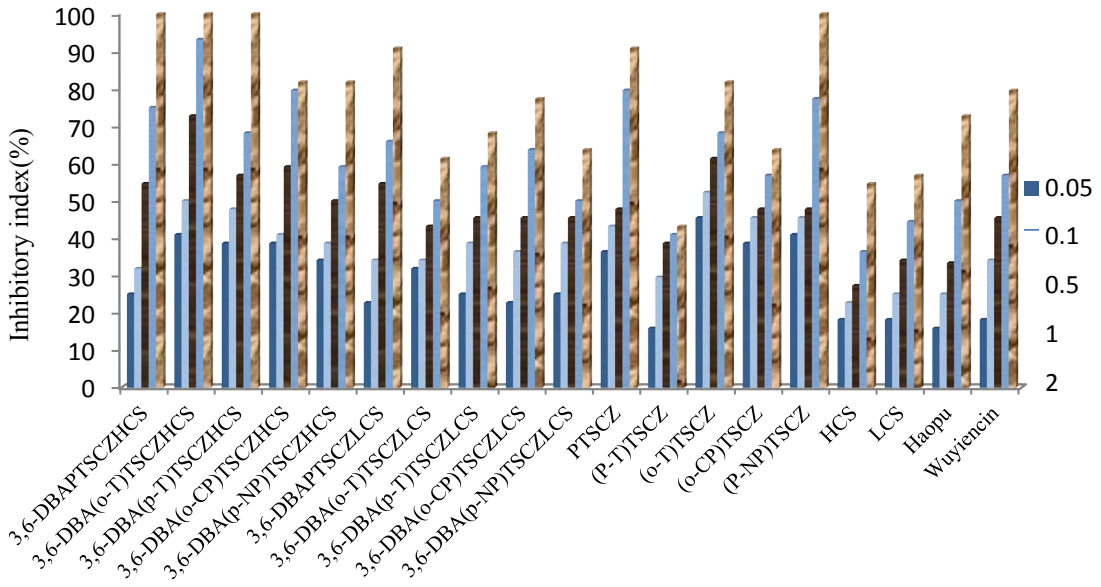

Figure 7: Antifungal activities of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *F. oxysporum f. sp. vasinfectum.*

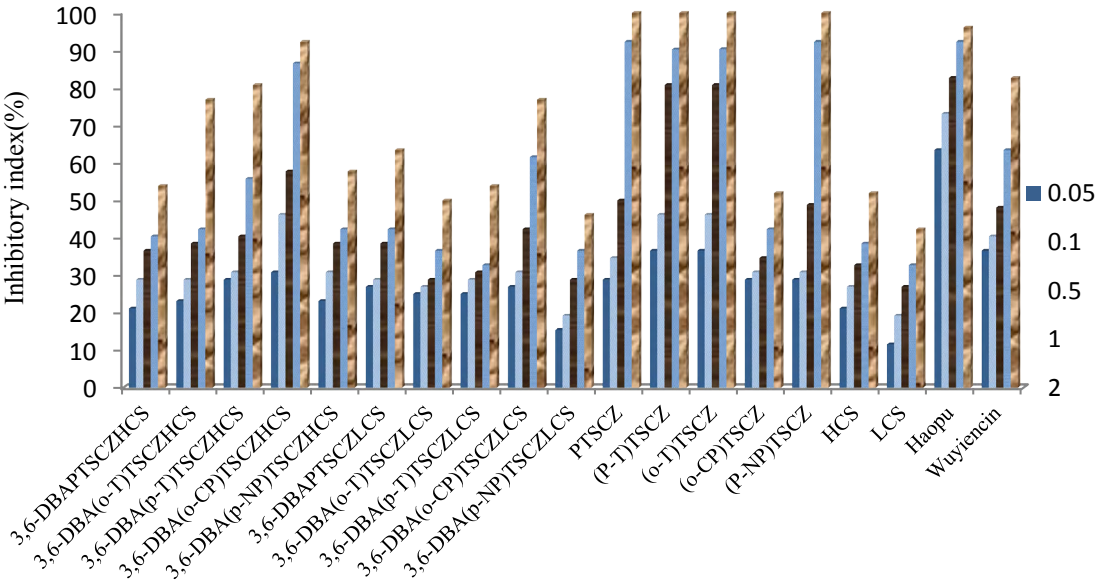

Figure 8: Antifungal activities of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan against *C. gloeosporioides (Penz.) Saec.*

reached 76.92%, 80.77%, 92.31%, 63.46%, 76.92%, respectively. The antifungal effect of 3,6-DBA(o-CP)TSCZHCS was better than that of positive control (Wuyiencin). It is generally believed that the antifungal action of Chitosan on fungi is related to the increase of cell membrane permeability and the germination of spores. Similar to the antibacterial mechanism of bacteria.

Zhong et al. [20] synthesized a group of acetyl phenyl-thiosemicarbazone-chitosan without locating protection in 2011. The antimicrobial effect of some $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan was improved by comparing with the acetyl phenyl-thiosemicarbazone-chitosan without locating protection. The MBC of all $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-Chitosan derivatives against *Escherichia coli* was reduced. The MIC and MBC of 3,6-DBAPTSCZHCS, 3,6-DBA(o-CP)TSCZHCS, 3,6-DBA(p-NP)TSCZHCS, 3,6-DBAPTSCZLCS, 3,6-DBA(o-CP)TSCZLCS and 3,6-DBA(p-NP)TSCZLCS on *Pseudomonas aeruginosa* were decreased. The MIC and MBC of all $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone-Chitosan derivatives against *Staphylococcus aureus* was decreased. The inhibitory effect of 3,6-DBA(o-CP)TSCZHCS, 3,6-DBA(p-NP)TSCZHCS, 3,6-DBA(o-

CP)TSCZLCS and 3,6-DBA(p-NP)TSCZLCS on *Alternaria solani* was improved. The inhibitory rate of 3,6-DBAPTSCZHCS, 3,6-DBA(o-CP)TSCZHCS, 3,6-DBA(p-NP)TSCZHCS, 3,6-DBA(o-CP)TSCZLCS and 3,6-DBA(p-NP)TSCZLCS against *F. oxysporum f. sp. vasinfectum* was increased. At a concentration of 0.5 $mg{\cdot}mL^{-1}$, the antifungal rate of 3,6-DBAPTSCZLCS against *F. oxysporum f. sp. vasinfectum* was increased. 3,6-DBAPTSCZHCS, 3,6-DBA(o-CP) TSCZHCS, 3,6-DBA(p-NP)TSCZHCS, 3,6-DBA(o-CP)TSCZLCS and 3,6-DBA(p-NP)TSCZLCS increased the inhibitory rate against *C. gloeosporioides (Penz.) Saec.* 3,6-DBA(p-NP)TSCZHCS, 3,6-DBA(o-CP)TSCZLCS and 3,6-DBA(p-NP)TSCZLCS increased the inhibitory rate against *F. oxysporum f. sp. cucumerinum*. The inhibitory effect of 3,6-DBAPTSCZHCS, 3,6-DBA(o-CP)TSCZHCS and 3,6-DBAPTSCZLCS on *F. oxysporum f. sp. cucumerinum* was better when concentrations were 0.05 $mg{\cdot}mL^{-1}$ and 0.1 $mg{\cdot}mL^{-1}$.

This result may be due to the substitution position and degree of substitution of the substituted group, the influence of the concentration of chitosan derivatives and the differences in the structure and drug resistance of the bacteria. From the experimental results, we can see that the microbial inhibitory effect of thiosemicarbazone crystal is much better than that of Chitosan. So, if the acetyl phenyl-thiosemicarbazone-chitosan is the product of the substitution reaction between 2, 3, 6 three sites of chitosan and thiosemicarbazone, its antimicrobial effect is superior to that of $C_{3,6}$-dibenzoylated phenyl-thiosemicarbazone derivatives of Chitosan. And the higher the degree of substitution, the better the antimicrobial effect. The antimicrobial activity of chitosan and its derivatives is closely related to its concentration, and the ability to resist microorganisms increases rapidly in a certain concentration range, and the anti-microbial action decreases or does not change when the concentration is above or below this range. Finally, different derivatives have different antimicrobial activity because of the difference of the structure and drug resistance of the strains.

Conclusion

According to the experimental results, the $C_{3,6}$-dibenzoylated phenyl- thiosemicarbazone derivatives of Chitosan have stronger antimicrobial effect than that of chitosan. Some derivatives have even more antimicrobial resistance than positive control drugs. The antimicrobial effects of the tested samples increased with increasing concentration. The antibacterial effect of derivatives on Gram negative bacteria is better than that of gram positive bacteria. Among the four plant pathogenic fungi, the derivatives had the worst inhibitory effect on *F. oxysporum f. sp. cucumerinum* and had the best antifungal effect against *F. oxysporum f. sp. vasinfectum*. 3,6-DBA(o-T)TSCZHCS, 3,6-DBA(p-T)TSCZHCS, 3,6-DBAPTSCZLCS and 3,6-DBA(o-CP) TSCZLCS have good antimicrobial effects on all tested strains, and have the value of further development.

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