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Research Article

The Anti-microbial Activity of C3,6-Dichloroacetyl Phenyl-Thio-semicarbazone-Chitosan Derivatives

Liu Yang, Zhimei Zhong*

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

*Corresponding author e-mail: zhimeihappy@126.com

ABSTRACT

In this paper, we study anti-microbial activity of ten kinds of C3,6-dichloroacetyl phenyl-thiosemicarbazone-chitosan derivatives against four species of bacteria and four fungi at different concentrations. And the mechanism of antimicrobial was discussed. The results showed that the antimicrobial activities of all derivatives were stronger than that of Chitosan. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the derivatives against *Escherichia coli* were 7.40 μg mL^{-1} and 29.16 μg mL^{-1} respectively. All of the derivatives had a definite antifungal effect on fungi in the concentration range of 0.05 $mg{\cdot}mL^{-1}$ to 2 $mg{\cdot}mL^{-1}$, with a maximum inhibition index of 100%. The results showed that the antibacterial abilities of the derivatives have potential value in the field of new pesticide development.

Keywords: Anti-bacterial activity, Anti-fungal activity, Chitosan derivatives, Dichloroacetyl phenyl-thiosemicarbazone

INTRODUCTION

Chitosan is a natural basic polysaccharide polymer, made up of β-(1,4)-linked 2-amido-2-deoxy-d-glucopyranose and β-

(1,4)-linked 2-acetamido-2-deoxy-d-glucopyranose[1]. It is one of the most plentiful biomaterial with interesting characteristics, such as biodegradability, biocompatibility, antimicrobial activity, nontoxicity, versatile chemical and so on. Among the various characteristics of Chitosan, its antimicrobial activity is the most promising properties that have been found very fruitful in killing or inhibiting fungi and bacteria[2]. But its utilization in pharmaceutical is limited because original Chitosan is insoluble in neutral and alkaline pH conditions[3]. In order to overcome this drawback and get stronger antimicrobial activity, Chitosan derivatives by chemical modifications has becoming an active research topic. Zhong et al.[4] prepared 12 kinds of new hydroxyl benzene sulfonailides derivatives of Chitosan. Their water solubility and antimicrobial activities against four animal pathogenic bacteria and five crop-threatening pathogenic fungi are higher than that of unmodified Chitosan. Tamer et al.[5] prepared two aromatic Chitosan Schiff bases (I and II) via coupling with 4-chloro benzaldehyde and benzophenone. They tested their antimicrobial effects against three Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* sp.), two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and *Candida albicans* strain. The result of the derivatives for the test have shown that derivatives of Chitosan have better antimicrobial effects than Chitosan in most microorganisms. Sun et al.[6] via a facile chemical procedure by using 1,3-propane sultone attached to the backbone of Chitosan prepared sulfonated Chitosan and tested its antimicrobial activity. The result shows that sulfonated Chitosan exhibited higher antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* with the minimum inhibitory concentration (MIC) of 0.13 $mg \cdot mL^{-1}$ and 2.00 $mg \cdot mL^{-1}$ than those of water-soluble Chitosan with MIC of 0.50 $mg \cdot mL^{-1}$ and 4.00 $mg \cdot mL^{-1}$. Therefore, Chitosan derivatives using as antiseptic was researched with promising prospects.

Thiourea is an excellent disinfectant but has a strong toxicity. It has been confirmed that thiourea derivatives have a wide range of biological activities, such as anti-allergy, anti-inflammatory and antimicrobial activity etc. The antifungal activities of thiourea derivatives can compared with antifungal antibiotic ketoconazole[3]. Arslan et al.[7] have been synthesized five thiourea derivative ligands and their Ni^{2+} and Cu^{2+} complexes. All the derivatives can be inhibiting the

growth of microorganisms within lower scope of concentration. Krishna Reddy et al.[8] synthesized a series of novel 5-(3-cyano-4-isobutoxyphenyl)-4-methylthiazole-2-carbonyl-N-(substitutedphenyl) piper-azine-1-carboxamide/carbothiomide derivatives and obtained good results in against Tobacco mosaic virus and antimicrobial pathogens. Therefore, thiourea derivatives of Chitosan can not only improve the water solubility of Chitosan, but also reduce the toxicity of thiourea. In this paper, we studied the antimicrobial activity of ten C3,6-dichloroacetyl phenyl-thio semicarbazone-chitosan derivatives against four species of pathogenic bacteria and pathogenic fungi of four plants, and the results were compared with that of the original Chitosan and phenyl-thio semicarbazone crystal. On the basis of this, we hope to find several active derivatives as prodrug compounds, which will have laid the foundation for the next step to prepare new type of antibacterial agricultural fungicides.

EXPERIMENTAL PROCEDURE

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. cucumerinum* (BNCC 227992), *C. gloeosporioides* (Penz.) *Saec* (BNCC 114936) and *F. oxysporum, F. vasinfectum* (BNCC226606), *Escherichia coli* (BNCC 336902), *Micrococcus luteus* (BNCC 102589) was purchased from BeNa Culture Collection. *Staphylococcus aureus* (FSCC223001) and *Pseudomonas aeruginosa* (FSCC 206003) was purchased from Guangdong Huankai Microbial Sci. and Tech. Co., Ltd.

Anti-bacterial assays

The minimum inhibitory concentration (MIC) refers to the minimum drug concentration that can inhibit the apparent growth of a certain microbe by incubation for 24 hours in a given environment. The minimum bactericidal concentration (MBC) is the lowest concentration of the drug that can kill 99.9% of the tested microorganisms[9]. All the derivatives were dissolved in 100 μg mL^{-1} HAC. The MIC and MBC was determined by the stepwise dilution method in a 96-well microtiter plates with a single pore volume of 400 μL. We choose twelve concentration gradients (3789.6, 1894.8, 947.4, 473.7, 236.8, 118.4, 59.21, 29.61, 14.80, 7.40, 3.70, 1.85 μg·mL^{-}

^{1}) in this study. Firstly, 180 µL medium was added to the 1-12 hole. Then added 180 µL sample solution to the first hole, mixed evenly and moved into the second hole, and so on. 180 µL was removed from the twelfth hole. Finally, each hole added 20 µL bacteria liquid, select a column without sample as blank control. The above operations are carried out in the clean bench. Norfloxacin was used as a positive control, and three groups of drugs were used in parallel experiments. Place the culture plate in an incubator, cultivated for 24 - 48 h at 37°C. The OD value of the sample was determined by the enzyme analyzer and the bacteriostatic rate was calculated as follows:

Antimicrobial rate (%) = (ODb-ODa) / ODb

ODa is the OD value of the sample, ODb 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.

Anti-fungal assays

Antifungal assays were performed based on the method of Zhong et al.[10]. The experiment was conducted to test the inhibitory effect of five samples concentrations on *Alternaria solani*, *F. oxysporum F. cucumerinum*, *C. gloeosporioides* (Penz.) *Saec* and *F. oxysporum F. vasinfectum*. Firstly, the 37.6 µL, 75 µL, 385 µL, 789 µL, 1670 µL sample solution were added to 15 mL medium, and the medium drug content was 0.05 mg·mL^{-1}, 0.1 mg.mL^{-1}, 0.5 mg·mL^{-1}, 1 mg·mL^{-1}, 2 mg·mL^{-1}. Then, the culture medium was evenly poured into a petri dish with a diameter of 9 cm, and 2 pieces of fungus with a diameter of 4 mm were inoculated in each culture dish after medium being completely solidified. After the culture of 48 h or 72 h at 29°C, the colony diameter was measured, and the inhibitory index of the sample was calculated. All operations are performed in the clean bench and three groups of drugs were used in parallel experiments. The experiment was conducted with the same concentration of Wuyi and Haopu oligosaccharides as positive control and distilled water as negative control. The inhibitory index was calculated as follows:

Inhibitory index (%) = (Db-Da)/(Db-4) × 100

Da is the diameter of the growth zone in the test plate and Db 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 antibacterial activity of C3,6-dichloroacetyl phenyl-thiosemicarbazone-chitosan derivatives was compared with that of CS, TSCZ, and Norfloxcin in the experiment. The results indicated that all of the derivatives showed higher antibacterial activity than CS, and some of them show stronger activities than TSCZ. The minimum value of MIC and MBC of the derivatives against *Eschercichia coli* was 7.40 μg·mL^{-1} and 29.61 μg·mL^{-1} respectively. It can be seen from Table 1 and Figures 1-4 that the inhibitory effect of derivatives on Gram negative bacteria is stronger than that of Gram positive bacteria. The main reason for this result may be due to the antibacterial mechanism of Chitosan with different molecular weight, the difference of the cell wall structure of gram negative and positive bacteria, and the degree of derivatives. The cell wall of Gram positive and negative bacteria is different, so the inhibition mechanism of Chitosan and its derivatives for Gram positive and negative bacteria is also different[11]. The cell wall of Gram negative bacteria is a double layer. In addition to the cell membrane and cell wall, the outer layer of the negative bacteria cell membrane is also characterized by a unique outer membrane[10-12]. The cell wall of Gram negative bacteria is composed of a thin peptidoglycan membrane. The outer cytoplasmic membrane is composed of lipopolysaccharide, lipoprotein and phospholipids stabilized by divalent cations such as Mg^{2+} and Ca^{2+}. If the Chitosan is protonated, the surface of the carboxyl group and the phosphate group of the bacteria are anionic, which provides a potential site for the electrostatic binding of Chitosan. The permeability of cell wall changed and the osmotic stability decreased. The derivatives destroy the barrier properties of the membrane, enters the cell, interferes with physiological activity or leaks from bacterial enzymes and nucleotides[13]. The lower the molecular weight, the easier it is to enter the void structure of the cell wall, interfering with the metabolism of cells. Therefore, the inhibitory effect of low molecular weight Chitosan on Gram negative bacteria was stronger. The cell wall of Gram positive bacteria is mainly composed of peptidoglycan, which does not allow the formation of outer membrane. It is speculated that the main anti-bacterial

mechanism of Gram positive bacteria is based on macromolecular Chitosan formation of a polymer membrane, blocking the nutrients into the surface of the polymer membrane; the drug can prevent nutrients into the cell or leakage[14]. Thus, high molecular weight Chitosan has good antibacterial effect against gram positive bacteria. However, due to the different degree of substitution, the effect of the substituted groups on the antibacterial effect of Chitosan is different[15]. Therefore, the antibacterial effects of these ten derivatives do not fully comply with the above mechanism. In general, the antibacterial effect of 3,6-DCA(O-T) TSCZHCS, 3,6-DCA(p-T)TSCZHCS, 3,6-DCA(o-CP)TSCZHCS and 3,6-DCA(p-NP)TSCZHCS was remarkable.

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

The most important seeds or plant diseases in agriculture are caused by fungi. So, we need to find safe, nontoxic and environmentally friendly antimicrobial agents to protect crops[16]. *Alternaria solani* is one of the most serious tomato diseases in the world. It is transmitted through air borne, soil inhabiting and is responsible for early blight, collar rot and fruit rot of tomato[17]. *Fusarium oxysoporum* and *F. vasinfectum* can cause cotton blight, which is extremely damaging to high-quality cotton production[18]. C. *gloeosporioides* (Penz.) Saec can cause anthrax, a major post-harvest disease that affects many fruits and crops[19]. *Fusarium oxysporum* and *F. cucumerinum* is the fungal pathogen responsible for *Fusarium* vascular wilt of cucumber. In Australia, the growth of Cucumber in the soilless greenhouse is threatened by the disease[20].

Figures 5-8 show the results of the derivatives against *Alternaria solani*, *F. oxysporum F. cucumerinum*, *C. gloeosporioides* (Penz.) *Saec* and *F. oxysporum* and *F. vasinfectum*. All derivatives have strong antifungal effect, this may be related to the increase of cell membrane permeability and the influence on spore germination. The antifungal activity of all compounds is superior to that of chitosan, and the antifungal effect of most macromolecule chitosan compounds is stronger than that of low molecular weight chitosan compounds. For different strains, the antibacterial effect of drugs is also different. 3,6-DCA(o-T)TSCZHCS, 3,6-

DCA(p-T)TSCZHCS and 3,6-DCA(p-NP)TSCZHCS has better antibacterial effect against *Alternaria solani*. When the concentration was 2 mg·mL^{-1}, the inhibitory rates were 77.78%, 74.07%, 74.07%, which was similar to positive comparing reagents. 3,6-DCA(p-T)TSCZHCS had the best antifungal effect on *F. oxysporum F. cucumerinum* and the inhibition rate was 90.74%, which was better than two kinds of positive comparing reagents. The inhibitory rate of 3,6-DCA(p-NP)TSCZHCS against *F. oxysporum F. cucumerinum* was 72.22%, better than that of positive comparing reagent (Haopu). For *F. oxysporum F. vasinfectum*, the inhibitory rate of 3,6-DCA(o-T)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS, 3,6-DCA(o-T)TSCZLCS reached 90.91%. When the concentration was 1 mg·mL^{-1}, the inhibitory rate of 3,6-DCA(p-T)TSCZHCS, 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-T)TSCZLCS reached 90.91%. And when their concentration was 2 mg·mL^{-1}, the rate of inhibiting fungi reached 100%, obviously more than positive comparing reagent. For *C. gloeosporioides* (Penz.) Saec, 3,6-DCA(o-T)TSCZHCS, 3,6-DCA(p-T)TSCZHCS, 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS has better antifungal effect, the inhibitory index was 92.31%, 84.62%, 80.77% and 80.77% respectively.

Compared with the chloracetyl phenyl-thio-semicarbazone-chitosans synthesized by Zhong et al.[16] without location protection, the antimicrobial activity of some C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan was improved. The MBC of 3,6-DCAPTSCZHCS, 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS and 3,6-DCAPTSCZLCS against *Escherichia coli* was decreased. The MBC of 3,6-DCAPTSCZHCS, 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS, 3,6-DCAPTSCZLCS, 3,6-DCA(o-CP)TSCZLCS, 3,6-DCA(p-NP)TSCZLCS against *Pseudomonas aeruginosa* was decreased. The MIC of 3,6-DCA(o-CP)TSCZHCS and 3,6-DCA(p-NP)TSCZHCS against *Pseudomonas aeruginosa* was decreased. The MIC of 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS, 3,6-DCAPTSCZLCS and 3,6-DCA(o-CP)TSCZLCS against *Staphylococcus aureus* was decreased. The MBC of 3,6-DCAPTSCZHCS, 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS, 3,6-DCAPTSCZLCS and 3,6-DCA(o-CP)TSCZLCS against *Staphylococcus aureus* was decreased. The MIC and MBC

of 3,6-DCA(o-CP)TSCZHCS against *Micrococcus luteus* were decreased. The inhibitory index of 3,6-DCA(p-NP)TSCZHCS, 3,6-DCAPTSCZLCS, 3,6-DCA(o-CP)TSCZLCS, 3,6-DCA(p-NP)TSCZLCS against *Alternaria solani* was increased. The inhibitory index of 3,6-DCAPTSCZHCS, 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS, 3,6-DCA(o-CP)TSCZLCS and 3,6-DCA(p-NP)TSCZLCS against *F. oxysporum F. vasinfectum* was increased. The inhibitory index of 3,6-DCA(p-NP)TSCZHCS and 3,6-DCA(p-NP)TSCZLCS against *C. gloeosporioides* (Penz.) *Saec* was increased. And at the concentrations of 0.05 $mg \cdot mL^{-1}$ and 0.1 $mg \cdot mL^{-1}$, the inhibitory index of 3,6-DCAPTSCZHCS against *C. gloeosporioides* (Penz.) *Saec* was increased. The inhibitory index of 3,6-DCA(o-CP)TSCZHCS, 3,6-DCA(p-NP)TSCZHCS and 3,6-DCA(p-NP)TSCZLCS against *F. oxysporum* and *F. cucumerinum* was increased. The main reasons for this result may be due to the different substitution sites and substitution degrees, the effect of concentration and the structure of the strain. As the antimicrobial effect of thiourea is very good, so if the part of the chloracetyl phenyl-thiosemicarba-zone-chitosans without location protection are the compounds that 2, 3, 6 three sites are reacted with the thiourea group, the antimicrobial effect of there are better than that of C3,6-dichloroacetyl phenyl-thio-semi carbazone derivatives of Chitosan. For compounds, the higher the degree of substitution, the better the antibacterial effect. The antimicrobial activity of chitosan and its derivatives have closely related to their concentration, and the inhibition rate increases rapidly in a certain concentration range, when the concentration above or below this range, the antimicrobial effect would have decreased or not changed. Finally, because of the differences in the structure and resistance of the strains, the antimicrobial activity of different derivatives is different.

CONCLUSION

In this study, the antimicrobial activity of ten new C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against four species of animal pathogenic bacteria and four crop-threatening pathogenic fungi at different concentrations was studied and compared with that of CS, TSCZ, positive comparing reagents. The results showed that the antimicrobial activity of ten derivatives was higher than that of Chitosan and the antifungal activity of some derivatives was higher than that of the

positive ones. The effects of antimicrobial activity were affected by the kinds of microorganism, substituting degree of derivatives, molecular weight of chitosan and the concentration of the tested sample. The anti-microbial activity of derivatives against gram negative bacteria is higher than that of gram positive bacteria, and the antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* such common strains is higher than *Pseudomonas aeruginosa*, *Micrococcus luteus* such resistant bacteria. For fungi, the rate of inhibition increases with the concentration of the drug. The inhibitory effect on *F. oxysporum F. vasinfectum* was better than that of other three fungi's.

These four derivatives, 3,6-DCA(o-T)TSCZHCS, 3,6-DCA(p-T)TSCZHCS, 3,6-DCA(o-CP)TSCZHCS and 3,6-DCA(p-NP)TSCZHCS have good inhibitory effects on most of the tested microorganisms and have high research value on the research and development of pesticides and veterinary drugs.

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Figures and Tables

Figure 1: The MIC and MBC of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of chitosan against *Escherichia coli*.

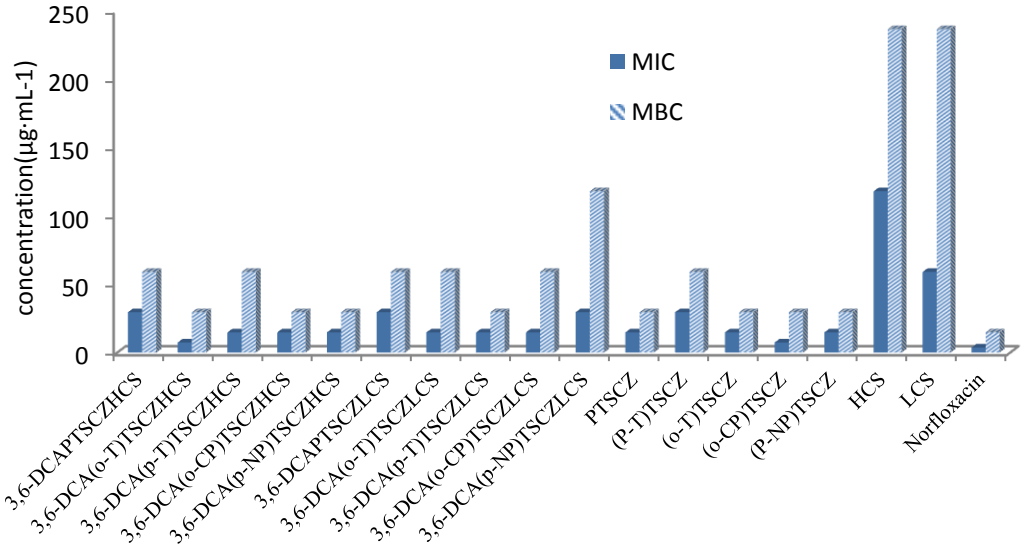

Figure 2: The MIC and MBC of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *Staphylococcus aureus.*

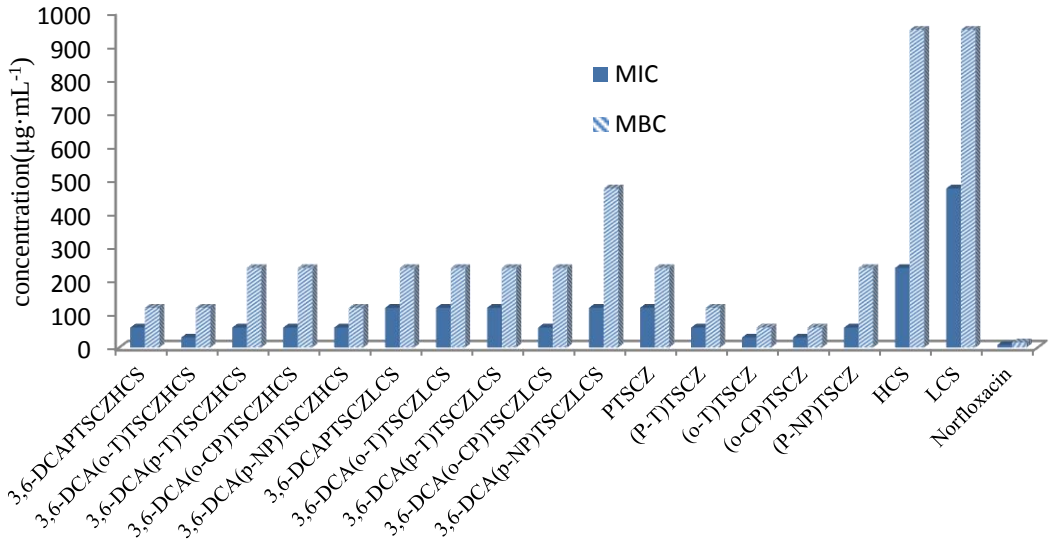

Figure 3: The MIC and MBC of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *Pseudomonas aeruginosa.*

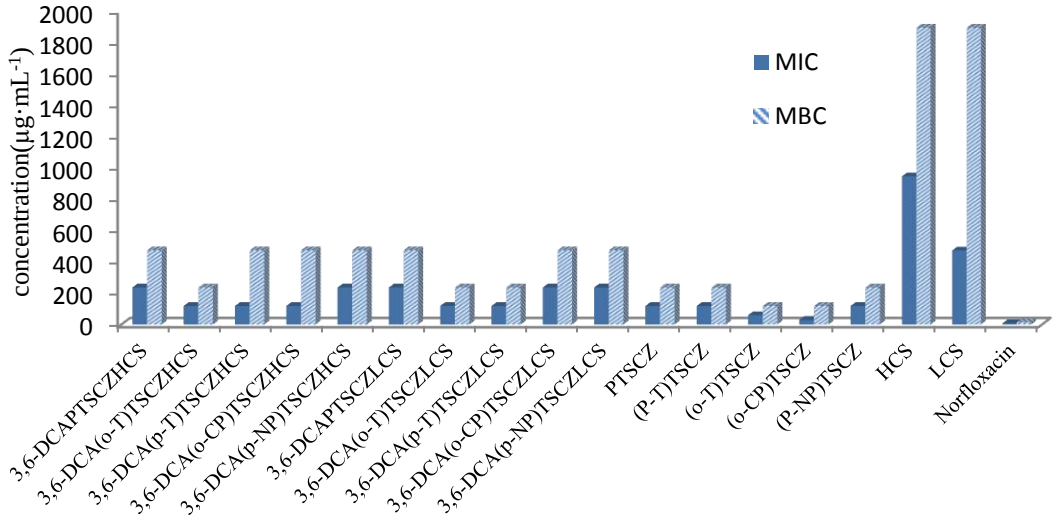

Figure 4: The MIC and MBC of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *Micrococcus luteus.*

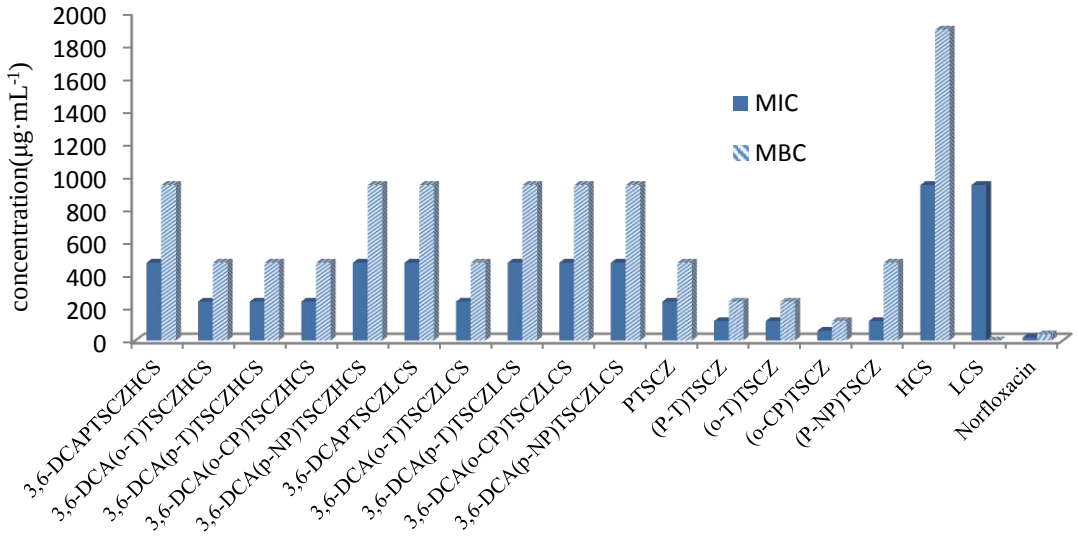

Figure 5: Antifungal activities of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *Alternaria solani.*

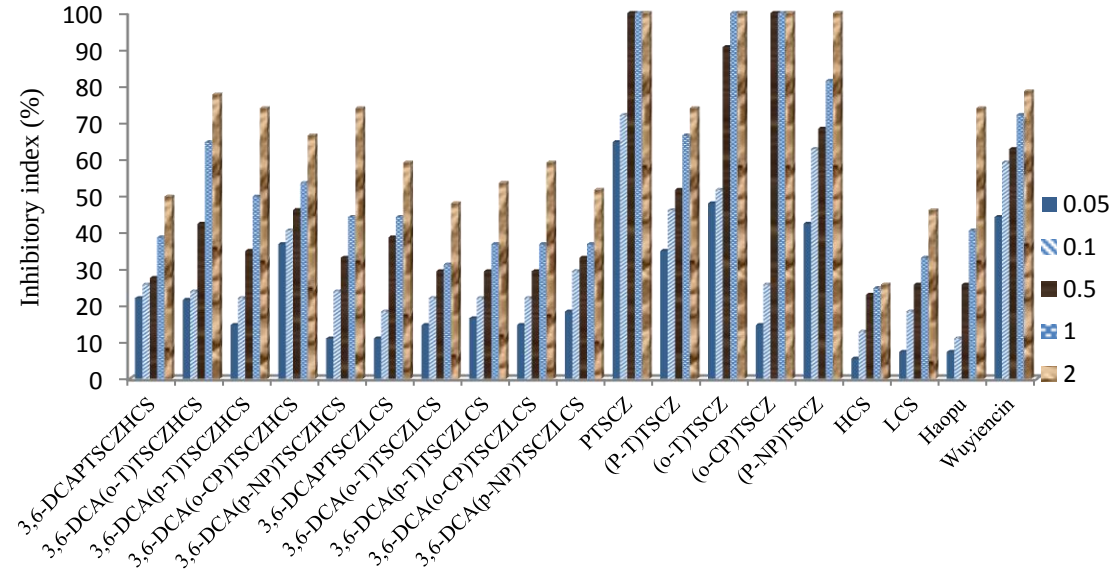

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Figure 6: Antifungal activities of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *F. oxysporum and F. vasinfectum.*

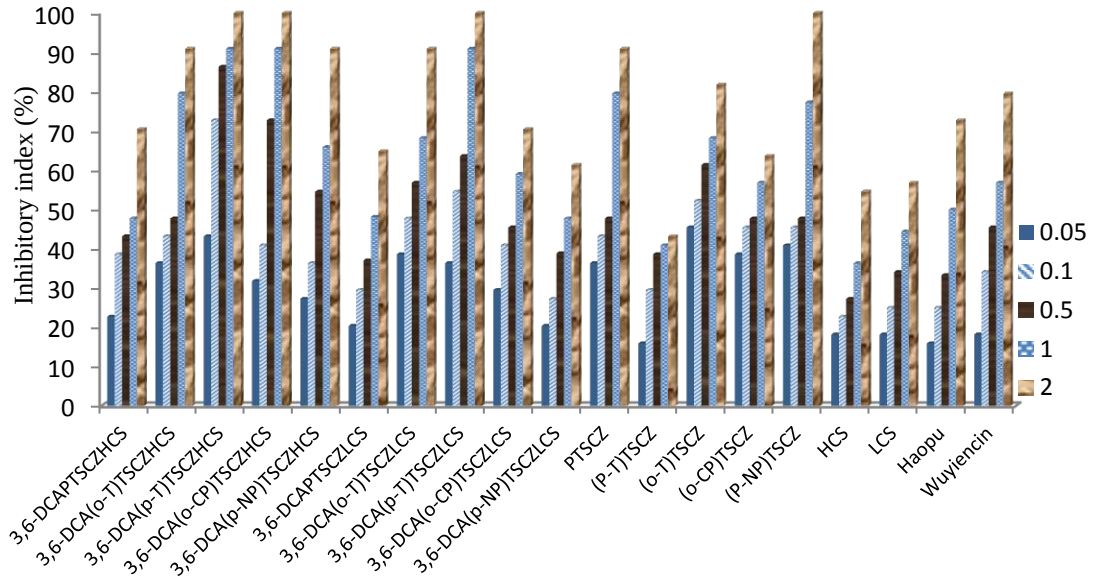

Figure 7: Antifungal activities of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *C. gloeosporioides (Penz.) Saec.*

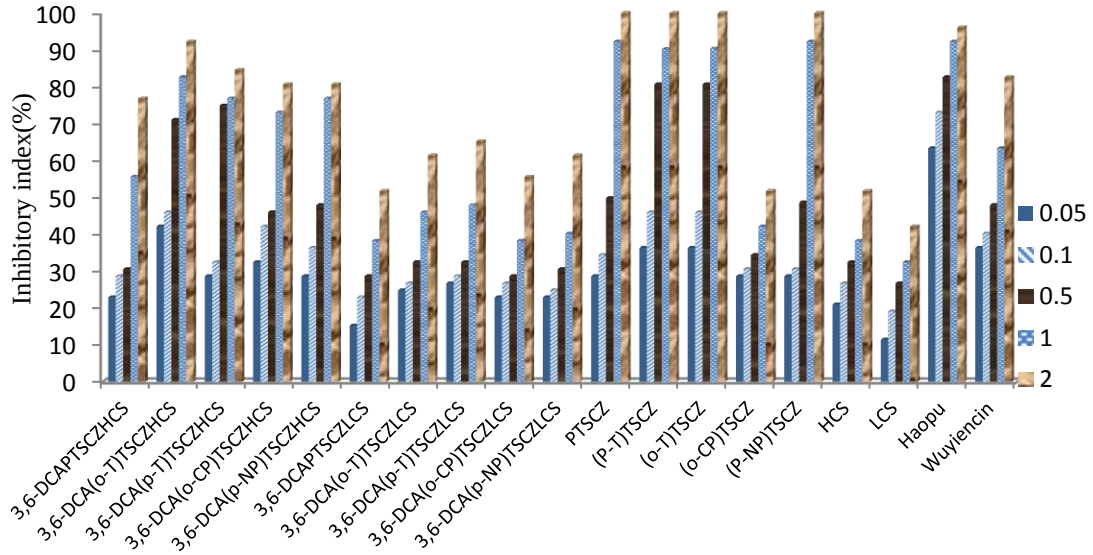

Figure 8: Antifungal activities of C3,6-dichloroacetyl phenyl-thiosemicarbazone derivatives of Chitosan against *F. oxysporum and F. cucumerinum.*

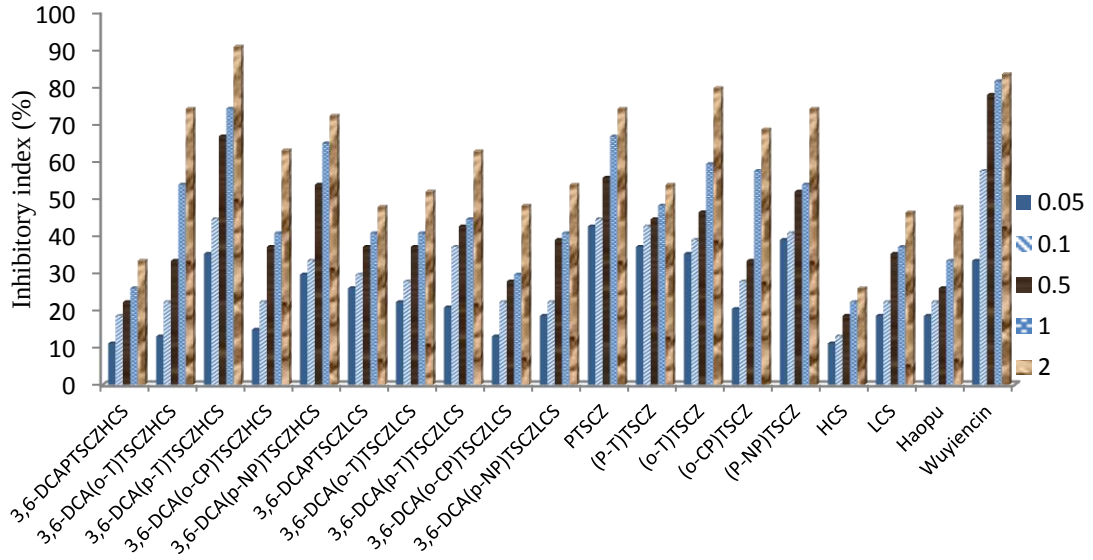

Table 1: MIC and MBC values of the samples against *Eschercichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Micrococcus luteus.*

Samples	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-DCAPTSCZHCS	236.8, 473.7	473.7, 947.4	29.61, 59.21	59.21, 118.4
3,6-DCA(o-T) TSCZHCS	118.4, 236.8	236.8, 473.7	7.40, 29.61	29.61, 118.4

3,6-DCA(p-T) TSCZHCS	118.4, 473.7	236.8, 473.7	14.80, 59.21	59.21, 236.8
3,6-DCA(o-CP) TSCZHCS	118.4, 473.7	236.8, 473.7	14.80, 29.61	59.21, 236.8
3,6-DCA(p-NP) TSCZHCS	236.8, 473.7	473.7, 947.4	14.80, 29.61	59.21, 118.4
3,6-DCAPTSCZLCS	236.8, 473.7	473.7, 947.4	29.61, 59.21	118.4, 236.8
3,6-DCA(o-T) TSCZLCS	118.4, 236.8	236.8, 473.7	14.80, 59.21	118.4, 236.8
3,6-DCA(p-T) TSCZLCS	118.4, 236.8	473.7, 947.4	14.80, 29.61	118.4, 236.8
3,6-DCA(o-CP) TSCZLCS	236.8, 473.7	473.7, 947.4	14.80, 59.21	59.21, 236.8
3,6-DCA(p-NP) TSCZLCS	236.8, 473.7	473.7, 947.4	29.61, 118.4	118.4, 473.7

[a] Not determined under the test condition.