Synthesis and characterization of some novel antimicrobial thiosemicarbazone O-carboxymethyl chitosan derivatives

Nadia A. Mohamed*, Riham R. Mohamed, Rania S. Seoudi

Department of Chemistry, Faculty of Science, Cairo University, Giza, Egypt

ABSTRACT

Three novel thiosemicarbazone O-carboxymethyl chitosan derivatives were obtained via a condensation reaction of thiosemicarbazide O-carboxymethyl chitosan with o-hydroxybenzaldehyde, p-methoxybenzaldehyde, and p-chlorobenzaldehyde respectively. Their structures were characterized by elemental analysis, FTIR, 1H NMR and X-ray diffraction. The antimicrobial behaviors of the prepared derivatives against three types of bacteria Staphylococcus aureus (S. aureus, RCMB A 2004), Bacillus subtilis (B. subtilis, RCMB 6005), and Escherichia coli (E. Coli, RCMB 50 03) and three crops-threatening pathogenic fungi Aspergillus fumigatus (A. fumigatus, RCMB 06002), Geotrichum candidum (C. candidum, RCMB 05098), and Candida albicans (C. albicans, RCMB 05035) were investigated. The results indicated that the antibacterial and antifungal activities of the investigated derivatives are much higher than those of the parent O-carboxymethyl chitosan. They were more potent in case of Gram-positive bacteria than Gram-negative bacteria. The presence of electron withdrawing chlorine atom on the aryl moiety of the aldehyde portion improved greatly antimicrobial activity to be nearly equivalent to the used standard drugs.

1. Introduction

Chitosan is mainly obtained from partial deacetylation of the second abundant natural polymer chitin. Chitosan, a polysaccharide consisting of β-(1,4)-linked 2-amido-2-deoxy-

d-glucopyranose and β-(1,4)-linked 2-acetamido-2-deoxy-
d-glucopyranose, has attracted great attention due to a better understanding of its inherent biological and physicochemical characteristics.

Aiming from its non-toxicity, biodegradability, biocompatibility, antimicrobial activity, versatile chemical and physical properties, chitosan has been applied in a variety of fields, such as medical applications, biotechnology, textiles, wastewater treatment, cosmetics, and agriculture [1].

Chitosan has been investigated as an antimicrobial material against a wide range of target microorganisms like; algae, bacteria, yeasts, and fungi in experiments involving in vivo and in vitro interactions with chitosan in different forms (solutions, films, and composites). The bacterial effectiveness on Gram-positive or Gram-negative bacteria is however, somewhat controversial. Some authors have stated that chitosan generally showed stronger effects for Gram-positive bacteria (e.g., L. monocytogenes, B. megaterium, B. cereus, S. aureus, L. plantarum, L. brevis, L. bulgaris, ... etc.) than Gram-negative bacteria (e.g., E. coli, P. fluorescens, S. typhimurium, V. paraaerohalodenica, ... etc.) [2,3]. Conversely, it has been demonstrated that hydrophilicity in Gram-negative bacteria is significantly higher than in Gram-positive bacteria, making them most sensitive to chitosan [4]. It was also proven that chitosan has a broad-spectrum antifungal activity against a variety of fungi [5,6]. However, these activities are limited to acidic conditions due to its poor solubility above pH 6.5. Recent studies have focused on the preparation of chitosan derivatives soluble in water, such as hydroxyethyl acryl chitosan [7], ethylamline hydroxyethyl chitosan [8], hydroxypropyl chitosan [9], and O-carboxymethyl chitosan (CMCs) [10].

CMCs, an amphoteric material with high hydrophilic characteristics, can be obtained through the reaction of chitosan with monochloroacetic acid in the presence of sodium hydroxide [11]. It is a very important chitosan derivative showing very good water solubility.

Some attempts have been made to further improve the already known antibacterial and antifungal activity of CMCs via grafting CMCs with N-vinylimidazole [12] and with N-

carbonyl-N’-cyanoacetoxydrazide [13], by crosslinking CMCs with terephthaloyl disothiocyanate [14] and via reacting of CMCs with acetyl, chloroacetyl and benzoyl isothiocyanate derivatives [15], and with various benzaldehyde derivatives [16].

* Corresponding author at: Department of Chemistry, College of Science and arts, Qassim University, Buraidah, KSA.
E-mail address: namadm@hotmail.com (N.A. Mohamed).
Thiosemicarbazones were well documented for their antibacterial, antifungal, antiviral, antitumor, and antimarial activities \cite{17, 18}. Recently, Qin et al., \cite{19} have prepared three thiosemicarbazone derivatives based on chitosan as a matrix with a low degree of substitution in the range of 8.62–11.20 and have tested these derivatives only as antifungal agents against \textit{S. solani}, \textit{R. solani}, \textit{A. solani}, and \textit{P. asparagus}.

In this study an attempt to combine the O-carboxymethyl chitosan with thiosemi-carbazones has been done to prepare more potent antimicrobial and antifungal thiosemicarbazone O-carboxymethyl chitosan derivatives. Our selection for O-carboxymethyl chitosan to be a matrix in our work is based on its good solubility both in aqueous and organic media in addition to its better antimicrobial activity than that of chitosan itself. Thus, it would be expected that the thiosemicarbazone derivatives of O-carboxymethyl chitosan may be obtained with a higher degree of substitution and will show better antimicrobial activity than that of the same derivatives based on chitosan. The thiosemicarbazone carboxymethyl chitosan derivatives prepared in this work will be tested as antibacterial agents against \textit{S. aureus} and \textit{B. subtilis} as Gram-positive bacteria and against \textit{E. coli} as gram-negative bacteria and as antifungal agents against \textit{A. fumigatus}, \textit{G. candidum}, and \textit{C. albicans}.

2. Materials and methods

2.1. Materials

Chitosan (code KB-002) was purchased from Funakoshi Co. Ltd., Japan. Its deacetylation degree is 88% and its average molecular weight is 190,000–310,000 Da. Sodium hydroxide, monochloroacetic acid, acetic acid, methanol, ammonium hydroxide, ethanol, carbon disulfide, sodium chloroacetate, hydrazine hydrate, \textit{p}-methoxybenzaldehyde, \textit{p}-chlorobenzaldehyde, and \textit{o}-hydroxybenzaldehyde were of analytical grade from Aldrich and were used as received. The crop-threatening pathogenic fungi \textit{Aspergillus fumigatus} (\textit{A. fumigatus}, RCMB 06002), \textit{Geotrichum candidum} (\textit{G. candidum}, RCMB 05098) and \textit{Candida albicans} (\textit{C. albicans}, RCMB 05035), and bacteria \textit{Staphylococcus aureus} (\textit{S. aureus}, RCMB 2004), \textit{Bacillus subtilis} (\textit{B. subtilis}, RCMB 6005), and \textit{Escherichia coli} (\textit{E. coli}, RCMB 5003) used for the antimicrobial assay were provided by the Regional Center for Mycology and Biotechnology Culture Collection.

2.2. Methods

2.2.1. Preparation of O-Carboxymethyl chitosan (CMCs)

O-Carboxymethyl chitosan (CMC) was prepared according to the method described by Chen and Park (2003) by stirring 5 g of chitosan in 100 mL aqueous NaOH solution (20% w/v) for 15 min. Twenty-five grams of monochloroacetic acid was added portion wisely to the alkalized chitosan and the reaction was continued at 40 °C for 2 h with continuous stirring. Then the reaction mixture was neutralized with 10% acetic acid, poured into an excess of 70% methanol, filtered using a G3 sintered funnel, and washed with methanol. The produced O-carboxymethyl chitosan was dried in a vacuum oven at 55 °C for 8 h to give 7.5 g dried O-carboxymethyl chitosan (Yield, 98.43%). The O-carboxymethylation process done on chitosan was proven by IR analysis (Sabaa et al., 2012). The degree of substitution (0.75) was determined according to the method described in literature \cite{20}.

2.2.2. Synthesis of thiosemicarbazone O-Carboxymethyl-chitosan (TCDCMCs) derivatives

Thiosemicarbazide O-carboxymethyl chitosan (TCDCMC) was prepared via one pot synthesis according to the method reported previously \cite{19}. A mixture of CMCs (16 g) and ammonium hydroxide (20 mL) was stirred in 95% ethanol for half an hour at room temperature. Then, carbon disulfide (8 mL) was slowly dropped into the mixture. After stirring for 2 h, ammonium dithiocarbamate CMCs was obtained. Next, sodium chloroacetate (11.5 g) was added into ammonium dithiocarbamate CMCs and keep the mixture reacting for 30 min to get sodium carboxy dithiocarbamate CMCs. At last, 85% hydrazide hydrate (12 mL) was slowly added to sodium carboxy dithiocarbamate at room temperature. When the reaction time has extended for another 2 h, the resulting mixture was filtered through filter funnel Büchner. The residue, a light brown powder, was washed with ethanol and dried at 60 °C to obtain thiosemicarbazide carboxymethyl chitosan (TCDCMCs) (Scheme 1).

TCDCMCs (10 mmol) were mixed with \textit{p}-methoxybenzaldehyde, \textit{p}-chlorobenzaldehyde or \textit{o}-hydroxybenzaldehyde (10 mmol) in methanol (100 mL). Then, acetic acid (0.2 mL) was added slowly. After refluxing for 10 h, the resultant mixture was

\begin{center}
\includegraphics[width=\textwidth]{Scheme1.png}
\end{center}

Scheme 1. Synthesis of thiosemicarbazide carboxymethyl chitosan (TCDCMCs) and thiosemicarbazone carboxymethyl chitosan (TCNMCs) derivatives.
cooled to room temperature and filtered through filter funnel Büchner. The precipitate was washed with methanol and dried at 60 °C to give p-methoxybenzaldehyde thiosemicarbazone CMCs (p-MeOBTCNCMCs), o-hydroxybenzaldehyde thiosemicarbazone CMCs (o-HOBTNCNCMCs), and p-chlorobenzenaldehyde thiosemicarbazone CMCs (p-Cl BTCNCMCs) (Scheme 1).

2.3. Measurements

Elemental analyses of the prepared derivatives were done in a Perkin–Elmer (Model 2410 Series II) C, H, N, S Analyzer (USA) at the Microanalytical Center, Cairo University (Egypt).

FTIR spectra were recorded using KBr discs on Testan Shimadzu IR-Spectrometer (FTIR model 8000) at room temperature within the wave number range of 4000–400 cm⁻¹.

XRD measurements of the powder samples were performed with a PAN analytical X'Pert powder. The scanning rate was 1.2°/min and the scanning scope of 2θ was 5–95°.

13C NMR spectra were recorded using a Varian Gemini–300 MHz instrument in DMSO–d6 as a solvent at 25 °C. Chemical shifts (δ) are expressed in parts per million (ppm) from tetramethylsilane as an internal standard.

The antimicrobial activity of CMCs, TCDCMCs and its TCNCMCs derivatives were evaluated against S. aureus (RCMBA 2004) and B. subtilis (RCMBA 6005) as Gram-positive bacteria and against E. coli (RCMBA 5003) as Gram-negative bacteria and against A. fumigatus (RCMBA 06002), G. candidum (RCMB 05098), and C. albicans (RCMB 05035) as fungi. Agar disk diffusion method was used for the determination of the antibacterial and antifungal activity, the well diameter was 6 mm (100 μL was tested), and the concentration of the tested sample was 5 mg/ml. Penicillin G, streptomycin, and amphotericin B were used as reference drugs against Gram-positive bacteria, Gram-negative bacteria, and fungi, respectively.

3. Results and discussion

3.1. Characterization of TCNCMC derivatives

Fig. 1 shows FTIR spectra of CMCs, TCDCMCs, o-HOBTNCNCMCs, p-MeOBTCNCMCs, and p-CIBTCNCMCs. The spectrum of CMCs showed, in addition to all the characteristic absorption peaks of chitosan, a new strong peak at 1412 cm⁻¹ which could be assigned to the symmetrical stretching vibration of COO⁻ group [21]. The asymmetrical stretching vibrating of COO⁻ group near 1550 cm⁻¹ is overlapped with the deforming vibration of NH₂ at 1600 cm⁻¹ to obtain a very strong peak. The C=O absorption peak of the secondary hydroxyl group became stronger and was shifted to 1074 cm⁻¹. The results indicated that the carboxymethylation process had occurred at C₂ position [22].

Compared with CMCs, TCDCMCs showed the appearance of a new band at 1634 cm⁻¹, which is attributed to the –NH–CS–NH– group. In addition, the peak corresponded to amide I at 1650 cm⁻¹ (C=O) has disappeared, the peak of the NH bending in the primary amine at 1463 cm⁻¹ became weak indicating that most of the amino group had been substituted [23]. A new peak at 1609 cm⁻¹ for the –NH– NH₂ bending was also observed. All of the above results showed the synthesis of TCDCMCs.

Compared with TCDCMCs, the new bands at 1625 cm⁻¹ (–C=O–), at 831 cm⁻¹ (phenyl), and at 1270 cm⁻¹ (–OCH₃) confirmed the chemical structure of p-MeOBTCNCMCs; while the absorption bands at 1623 cm⁻¹ (–C=O–) and at 834 cm⁻¹ (phenyl) indicated that o-HOBTNCNCMCs had been achieved. Also the absorption bands at 1620 cm⁻¹ (–C=O–), at 859 cm⁻¹ (phenyl), and at 820 cm⁻¹ (Cl–C) confirmed the structure of p-CIBTCNCMCs.

Fig. 2 shows the 13C NMR spectra of both CMCs and TCDCMCs. In CMCs spectrum, the signal due to carbon (C₁) (signal assigned to C₁ carbon in chitosan) is shifted to 115 ppm because of the electron-withdrawing effect of the carboxymethyl substituents.
As various different units occur in the structure of CMCs, many of the signals in the spectrum of chitosan appear split in the spectrum of CMCs. The signals at 64 ppm (C_2), 65 ppm (C_3), 100 ppm (C_4), 70 ppm (C_5), and 66 ppm (C_6) are detected. The signal around 176 ppm is assigned to carbonyl carbon C=O of carboxymethyl groups. The peak at 56 ppm is assigned to the methylene (−CH_2−) carbon [24]. For the TCDCMCs, the peak at 176 ppm may be caused by both the C=S and the C=O of carboxymethyl group.

On the other hand, Fig. 3 shows the 13C NMR spectra of the three TCNCMCs derivatives. Compared with TCDCMCs, new peaks for o-HOBTCNCMCs have appeared at 128–132 ppm, which can be attributed to carbons of phenyl ring; also a peak appeared at 167 ppm that can be attributed to carbon –N=CH– group. This is confirming the synthesis of o-HOBTCNCMCs.

Similarly for p-MeOBTCNCMCs, a new peak appeared at: 166 ppm is attributed to carbon of (−N=CH−) group, 126–131 ppm for carbons (Ph–H), and also another peak at 116 ppm has been attributed for (−OCH_3) group. The peak at 166 ppm has been attributed to carbon (−N=CH−) group, 128–133 ppm assigned for carbons of phenyl and a characteristic peak at 136 ppm attributed to C–Cl, confirming the structure of p-CIBTCNCMCs [19].

An additional proof for the synthesis of the TCDCMCs and its TCNCMCs derivatives is given by their elemental analysis data as shown in Table 1. The elemental analysis results also
Table 1
Elemental analyses, % yield and % substitution degree of thiosemicarbazone O-carboxymethyl chitosan derivatives.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elemental analyses</th>
<th>% Yield</th>
<th>Substitution degree (%)</th>
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<tr>
<td></td>
<td>%C</td>
<td>%H</td>
<td>%N</td>
</tr>
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<td>8.43</td>
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<td>5.04</td>
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<tr>
<td>Soda. carboxy dithiocarbamate CMCs</td>
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<td>3.87</td>
<td>3.59</td>
</tr>
<tr>
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<td>5.03</td>
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</tr>
<tr>
<td>p-MeOBTCNCMCS</td>
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<td>5.04</td>
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</tr>
<tr>
<td>p-CIBTCNCMCS</td>
<td>45.86</td>
<td>4.38</td>
<td>9.08</td>
</tr>
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</table>

indicate that the substitution degree of TCDCMCs, o-HOBTCNCMCS, p-MeOBTCNCMCS, and p-CIBTCNCMCS are high enough and comparable (90.32, 87.50, 87.34, and 89.03, respectively) (Table 1). They are higher than those of the corresponding derivatives of chitosan (8.62–11.20) reported by Qin et al., (2012). This may be attributed to the better solubility of CMCs than that of chitosan. Further, all the prepared derivatives are produced in a quantitative yield (89–94%) (Table 1). The prepared derivatives are insoluble in all the common solvents except DMSO. The XRD patterns of CMCs, TCDCMCs, o-HOBTCNCMCS, p-MeOBTCNCMCS, and p-CIBTCNCMCS are shown in Fig. 4.

The characteristic diffraction peaks of CMCs appeared at 2θ around 16.5, 25, and 28° are attributed to its crystal form [25,26]. Spectrum of TCDCMCs was similar to that of CMCs, in addition to the new peaks appeared at 8, 2, 68, and 85°. It indicated that the crystalline structure of CMCs was slightly changed via chemical modification. This suggested that the original crystallinity of CMCs wasn’t destroyed. For o-HOBTCNCMCS, p-MeOBTCNCMCS, and p-CIBTCNCMCS, new peaks had appeared at 2θ = 6.4, 6.2, and 6.7°, respectively. Also, new sharp and high-intensive peaks for o-HOBTCNCMCS, p-MeOBTCNCMCS, and p-CIBTCNCMCS appeared at 2θ range of 15–30°. It suggested that the presence of thiosemicarbazone group forms a highly ordered structure [27].

Further, o-HOBTCNCMCS show characteristic peaks at 45 and 55°, as for p-MeOBTCNCMCS show characteristic peaks at 32 and 65°. These intensive peaks suggested that the presence of thiosemicarbazone group forms a highly ordered structure.

3.2. Antimicrobial activity

All of the synthesized substituted derivatives under investigation showed in vitro antimicrobial activity against the tested microorganisms. The results of antibacterial activity of the CMCs, TCDCMCs, and its TCNCMCS derivatives using inhibition zone method are listed in Table 2.

Compared with CMCs, TCDCMCs and its TCNCMCS derivatives have a higher antibacterial activity. Several mechanisms elucidating the antimicrobial activity of chitosan have been postulated. The most acceptable mechanism is mediated by the electrostatic forces between the protonated NH₃⁺ groups of chitosan and the electron-negative charges on the microbial cell surface [28]. As such mechanism is based on electrostatic interaction, it suggests that the greater the number of cationized amines, the higher will be the antimicrobial activity. Carboxymethylation of chitosan allowed the synthesis of CMCs with higher hydrophilicity, with better solubility in aqueous media and with greater positive charge density; where in CMCs the –COOH groups may react with the –NH₂ groups and changed these –NH₂ groups into –NH₃⁺ groups leading to increased polycationic character (non-pH dependent positive charges on CMCS). Further, the introduction of thiosemicarbazide and thiosemicarbazone moieties onto CMCS increases its cationic centers; thus, their NH and C–S groups can be protonated and consequently the net positive charge was strengthened, leading to a better antibacterial activity. Another proposed mechanism is the binding of chitosan with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via penetration of chitosan into the nuclei of the microorganisms [29]. Thiosemicarbazide and thiosemicarbazone moieties which are grafted onto the hydrophilic CMCS set the latter chains apart from each other, decrease their intermolecular hydrogen bonds, the reason for the ease of penetration of TCDCMCs and TCNCMCS derivatives into the cells of microorganisms and prevent the growth of the cell by preventing the transformation of DNA to RNA to obtain a higher antibacterial activity. The third mechanism is the chelating of metals, suppression of spore elements and binding to essential nutrients to microbial growth [30]. It is established that both the –COOH, thiosemicarbazide and thiosemicarbazone groups have an excellent metal-binding capacity [31]. This explains the observed higher antibacterial activity of TCDCMCs and TCNCMCS derivatives relative to that of the parent CMCS. Moreover, all the investigated derivatives showed a higher activity against the Gram-positive bacteria than against the Gram-negative bacteria (Table 2). As the strongest chloro derivative caused inhibition zone diameter of 24.3 ± 0.25 and 26.3 ± 0.67 mm for S. aureus and B. subtilis, respectively, corresponding to 23.1 ± 0.29 mm of E. coli. This may be attributed to their different cell wall. The results also reveal that the antibacterial activity is affected by the nature of the substituent group found in the aryl ring of TCNCMCS. A look at the results given in Table 2 directly reveals that the chloro derivative is characterized by greater antibacterial activity than that of the hydroxy and methoxy derivatives. This may be attributed to the electron withdrawing character of chlorine group that decreases the electron density at the thiosemicarbazone group, thus, increases its cationic character. The role of the electron withdrawing group in improving antibacterial activity is previously reported [32]. On the other hand, the hydroxy and methoxy are electron-rich substituent groups which can donate electrons toward the thiosemicarbazone group leading to decreasing its cationic character. It is worth mentioning that the chloro derivative has shown antibacterial activity against S. aureus and B. subtilis almost equivalent to that of the standard drug.
penicillin G and comparable to that of streptomycin against *E. coli*. The aliphatic derivative (TCDCMCs) showed lower antibacterial activity than that of the aromatic derivatives (TCNCMCs). Generally, compounds with aryl groups show more lipophilic character as compared to the compounds with aliphatic groups [33]. Thus, the aliphatic derivative does not penetrate into the bacteria as easily as the derivatives with aryl group do. This behavior can be attributed to the lower lipophilicity of the aliphatic derivative. The antifungal activities of CMCs, TCDCMCs and its TCNCMCs derivatives against *A. fumigatus* (RCMBA 06002), *G. candidum* (RCMB 05098), and *C. albicans* (RCMB 05035) are shown in Table 3. The results show that all the derivatives had effective activities against the tested fungi, compared with that of the parent CMCs, with inhibitory indices ranging from 25.5 ± 0.45 to 13.5 ± 0.59 mm inhibition zone (Table 3). Generally, chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation, and radial growth [34]. The antifungal mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells. The microscopic observation reported that chitosan molecules diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth [35]. Again, the results also demonstrate how the antifungal activities are affected by the nature of the substituent in the aryl ring of the TCNCMCs derivatives. Thus, the derivative with group of electron withdrawing nature (chloro derivative) showed higher inhibition zone diameter relative to that of having groups of electron donating nature (hydroxy and methoxy derivatives) (Table 3).

Furthermore, in case of *A. fumigatus*, the chloro derivative has shown almost equivalent activity to that of the standard drug amphotericin B, while in case of *C. albicans*, the chloro derivative has shown better activity than the standard drug amphotericin B. Finally, the aromatic derivatives (TCNCMCs) showed a higher antifungal activity than that of the aliphatic derivative (TCDCMCs).

### 4. Conclusion

Three new thiosemicarbazone *O*-carboxymethyl chitosan derivatives were prepared via one pot condensation reaction of thiosemicarbazide *O*-carboxymethyl chitosan with *p*-chlorobenzaldehyde, *o*-hydroxybenzaldehyde, and *p*-methoxybenzaldehyde. Different analyses were made to confirm the structures of the prepared derivatives like: FTIR, 13C NMR, elemental analyses, and XRD. Their antimicrobial activity against *S. aureus*, *B. subtilis*, and *E. coli* bacteria and *A. fumigatus*, *G. Candidum*, and *C. albicans* fungi were investigated. They were more potent in case of Gram-positive bacteria than Gram-negative bacteria. The inhibitory effect followed the sequence; *p*-CIBTCMCs □ *o*-HOBTCMCs □ *p*-MeOBTCMCs □ TCDCMCs □ CMCs. Additionally, the antimicrobial activity of the chloro derivative was almost as good as that of the used standard drugs. The derivative having electron withdrawing substituent group showed better antimicrobial activity than that of those containing electron donating ones.

### References
