Preparation, Characterization and Antimicrobial Activity of Carboxymethyl Chitosan Schiff Bases with Different Benzaldehyde Derivatives

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Abstract: Eighteen carboxymethyl chitosan (CMCh) Schiff bases and their reduced derivatives have been synthesized. They were characterized by spectral analyses (FT-IR and H^1-NMR) and scanning electron microscopy observation. Their antibacterial activities against *Streptococcus pneumonia*e (RCMB 010010)**,** *Bacillis subtilis* (RCMB 010067), as Gram positive bacteria and *Escherichia coli* (RCMB 010052) as Gram negative bacteria and the antifungal activity against *Aspergillus fumigatus* (RCMB 02568), *Geotricum candidum* (RCMB 05097), and *Candida albicans* (RCMB 05031) were examined using agar disk diffusion method. The results demonstrate how the antibacterial and the antifungal activity are clearly affected by both the nature and position of the substituent groups in the aryl ring of the prepared derivatives. CMCh-4-nitroBenz Schiff base and its reduced form show higher antimicrobial activity comparing with other para substituted derivatives. CMCh-4-nitroBenz Schiff base: 18.3, 17, and 15.6 mm against *Bacillis subtilis*, *Streptococcus pneumonia*, and *Escherichia coli* respectively and 16.2, 17.3, and 16.4 mm against *Aspergillus fumigates, Geotricum candidum,* and *Candida albicans* respectively. CMCh-4-nitroBenz reduced form: 19.5, 18.7, and 16.2 mm against *Bacillis subtilis*, *Streptococcus pneumonia*, and *Escherichia coli* respectively and 17.5, 19.5, and 17.4 mm against *Aspergillus fumigates, Geotricum candidum,* and *Candida albicans* respectively. Also CMCh-3-bromoBenz show good results; CMCh-3-bromoBenz schiff base: 19.2, 16.9, and 14.6 mm *Bacillis subtilis*, *Streptococcus pneumonia*, and *Escherichia coli* respectively and 18.4, 17.6, and 15.9 mm against *Aspergillus fumigates, Geotricum candidum,* and *Candida albicans* respectively.

[Nadia A. Mohamed, Magdy W. Sabaa, Ahmed HH. El-Ghandour, Marwa M. Abel-Aziz, Omayma F. Abdel-Gawad. **Preparation, Characterization and Antimicrobial Activity of Carboxymethyl Chitosan Schiff Bases with Different Benzaldehyde Derivatives.** *J Am Sci* 2013;9(3):247-264]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 49

Keywords: Carboxymethyl chitosan. Schiff bases. antibacterial activity. antifungal activity. minimum inhibitory concentration..

1. Introduction

Chitosan [poly-(β-1/4)-2-amino-2-deoxy-D-glucopyranose] is a collective name for a group of partially and fully deacetylated chitin compounds (Tikhonov *et al.,* 2006). Due to its unique biological characteristics, including biodegradability and nontoxicity, many applications have been found, either alone or blended with other natural polymers (starch, gelatin, alginates), in the food, pharmaceutical, textile, agriculture, water treatment and cosmetic Industries (Roberts, 1992; Arvanitoyannis *et al.,* 1998; Arvanitoyannis, 1999; Haque *et al.,* 2005; Kim *et al.,* 2005; Yamada *et al.,* 2005). The potential application of chitosan is hindered by its limited solubility in aqueous media. Thus, chitosan is chemically modified so as to improve its processability, solubility, antimicrobial activity and its ability to interact with other substances (Jayakumar and Selvamurugan, 2010). Introducing a carboxymethyl group is the most advantageous method of increasing the solubility of chitosan at neutral and alkaline pH media without affecting other important characteristics. *O*-carboxymethyl chitosan (CMCh) is an amphiprotic ether derivative of chitosan, containing both the –COOH and $–NH_2$ groups in the molecule, and possesses non-toxicity, biodegradability, biocompatibility, antimicrobial activity and has therefore received considerable attention in biomedical applications (Muzzarelli *et al.,* 1998; Jayakumar *et al.,* 2010). Chemical modification (Sun *et al.,*2006) of CMCh and physical blending with another polymer (Fan *et al.,* 2006) have been widely studied to obtain materials with novel properties.

Acrylic acid sodium salt (AASS) and methylacrylic acid sodium salt (MAASS) were grafted onto CMCh sodium salt (CMChS) to obtain copolymers (CMChS-AASS, and CMChS-MAASS) with good water solubility. Both copolymers express good antibacterial activities against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli)*, and CMChS-g-MAASS is more effective than CMCh-g-AAS (Xie *et al.,* 2002). Schiff bases characterized by

the -N=CH- (imine) groups are active against a wide range of organisms, including bacteria, fungi, and even algaes (Rehman *et al.,* 2004; Gu *et al.,* 2007; Slavica *et al.,* 2010; Varghese *et al.,* 2010). Schiff base containing imine groups can be prepared from the reaction between active carbonyl groups and amino groups. Some reports show that they have better physiological activities and applications in antibacterial, antiphlogistic and antiviral domain (Ispire 2009). Xueqiong *et al.*(2012) prepared seven Schiff bases from CMCh and para-substituted benzaldehydes, however, they only reported antibacterial activity of these Schiff bases against *E. coli* (ATCC 35218) and *S. aureus* (ATCC 25923) using the optical density method. Antibacterial activity of the Schiff bases was affected by the type of the substituent group at the para position of benzaldehyde.

In this study, we focused our attention on the synthesis of 18 CMCh Schiff bases using different benzaldehyde derivatives, containing either electron donating or electron withdrawing substituents. Reduction of the Schiff base derivatives was carried out using sodium cyanoborohydride ($NaCNBH_3$). All the CMCh derivatives were characterized through FT-IR, H^1-NMR. The derivatives surface was examined using scanning electron microscope (SEM). Antimicrobial activity of the samples against *Streptococcus pneumoniae* (*S. pneumonia*e) (RCMB 010010) and *Bacillis subtilis* (*B. subtilis*) (RCMB 010067) as Gram positive bacteria, and against *Escherichia coli* (*E. coli*) (RCMB 010052) as Gram negative bacteria and against *Aspergillus fumigatus* (*A. fumigatus*) (RCMB 02568), *Geotricum candidum* (*G. candidum*) (RCMB 05097) and *Candida albicans* (*C. albicans*) (RCMB 05031) fungi were measured through the diffusion agar technique. The influence of the nature, position and number of the substituent groups of the benzaldehyde on the antimicrobial activity was also discussed.

2. Materials and Methods:

Chitosan with a degree of deacetylation of 96%, and sodium hydroxide (Oxford laboratory Mumbai-400 002, India), monochloroacetic acid (ADWIC laboratory chemicals), sodium cyanoborohydride, 4-N,N-dimethylaminobenzaldehyde, 2-methoxybenzaldehyde, 2-thiophenecarboxyaldehyde, octaldehyde, and 3-bromobenzaldehyde (Aldrich), 4-hydroxybenzaldehyde, 4-nitrobenzaldehyde, 2,4-dichlorobenzaldehyde, 4-methoxybenzaldehyde, 4-methylbenzaldehyde, 2-chlorobenzaldehyde, 4-chlorobenzaldehyde, and 3,4-dimethoxybenzaldehude (Merck), 3-chlorobenzaldehyde (Koch-Light laboratories), 4-bromobenzaldehyde (Riedel-De Hcien), benzaldehyde, and anisaldehyde (BDH laboratory reagents), vanillin (NS. chemicals laboratory) were used in this study.

Experimental

Synthesis of CMCh

CMCh was prepared according to the method described by Chen and Park (2003) via stirring 5 g of chitosan in 100 ml of 20% NaOH (w/v) for 15 min. Then 15 g of monochloro acetic acid was added portion wisely to the reaction medium and stirring was continued for 2 hrs at 40°C. The reaction mixture was then neutralized with 10% acetic acid, poured into an excess of 70% methanol, filtered and washed with methanol. The produced CMCh sodium salt was dried in a vacuum oven at 55°C for 8 h. The sodium salt of CMCh was then acidified with a methanol solution containing nitric acid; the excess acid was then removed by washing with a methanol–water solution till acid free. The product was vacuum dried at 50 °C. The degree of substitution of CMCh was determined by pH titration (Liu *et al.,* 2004) and was found to be 0.75.

Synthesis of CMCh Schiff base derivatives

1.0 g of CMCh was dissolved in 30 ml of 1% (v/v) of acetic acid and then diluted with 50 ml EtOH. The CMCh solution was treated with the pre-determined amount of the aldehyde. The reaction mixture was then stirred at room temperature for 1 h and the pH of the medium was adjusted to be 5 by adding 1 M NaOH. Then, the product filtered and dried. In case of 4-nitrobenzaldehyde, DMF was used instead of EtOH.

Reduction of CMCh Schiff bases

NaCNBH3 1.026 g (16 mmol) was added to the previously prepared CMCh-Schiff base solution and was allowed to stir at room temperature for 24 hrs, followed by adjusting the pH to be 7 with 15% (w/v) aqueous NaOH. The solution was dialyzed with distilled water for 4 days. The product was isolated from the dialysis residue by lyophilization.

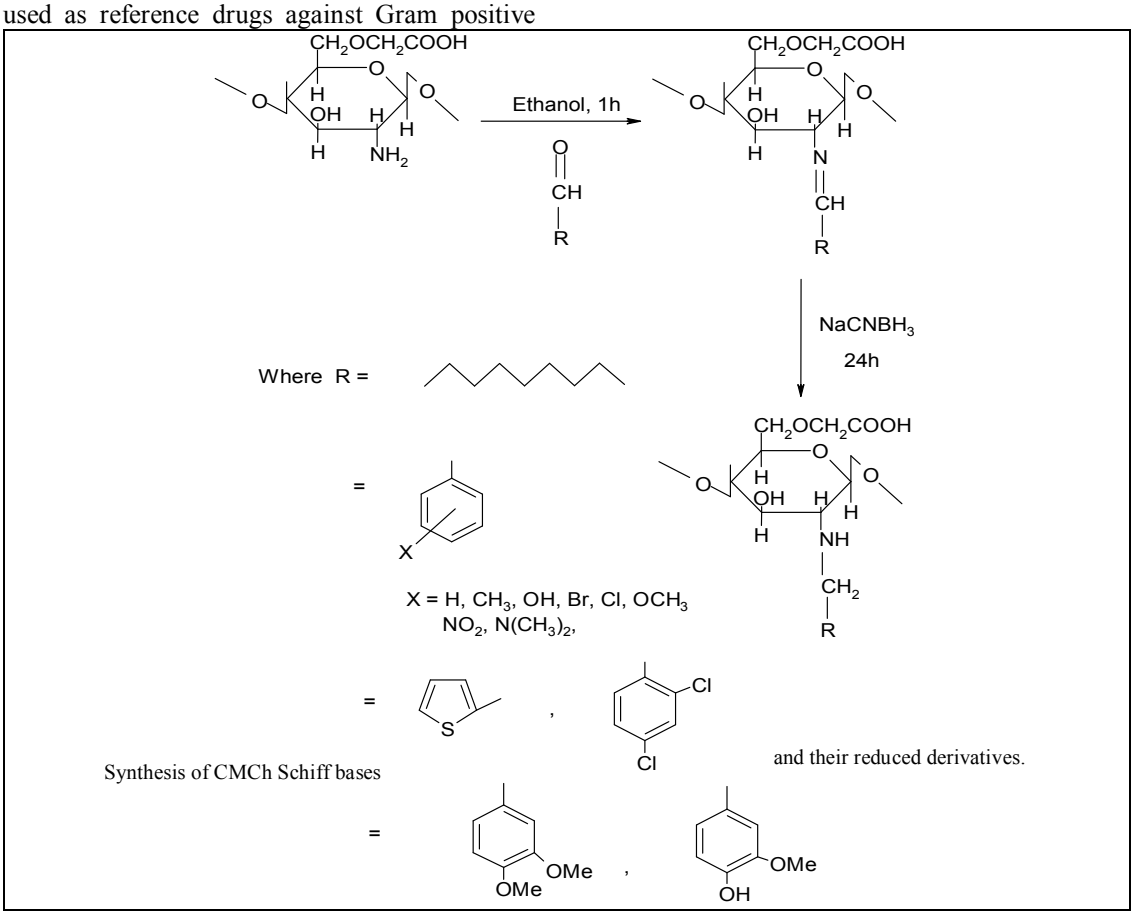
Characterization of the prepared CMCh derivatives

FTIR spectra were measured on spectrometer FT/IR, BRUKER Vector 22 (Germany), using KBr pellet technique. H^1-NMR spectra were recorded on Variam 300 MHz (USA). The surface of samples were examined with a scanning electron microscope (Inspect S company / FEI).

Antimicrobial measurements

The disks of Whatman filter paper were prepared with standard size (50 mm diameter) and kept into 10 screw capped wide mouthed containers for sterilization. These bottles are kept into hot air oven at a temperature of 150°C. Then, the standard sterilized filter paper disks impregnated with a solution of the test compound in DMSO (1 mg/mL) were placed on nutrient agar plate seeded with the appropriate test organism in triplicates. Standard conditions of 106 CFU/mL (Colony Forming U/mL) and 104 CFU/mL were used for antibacterial and antifungal assay, respectively. Petri dishes (9 cm in diameter) were used

and two disks of filter paper were inoculated in each plate. The utilized test organisms were *S. pneumoniae* (RCMB 010010) and *B. subtilis* (RCMB 010067) as examples of Gram positive bacteria and *E. coli* (RCMB 010052) as example of Gram negative bacteria. They were also evaluated for their *in vitro* antifungal potential against *A. fumigatus* (RCMB 02568), *G. candidum* (RCMB 05097) and *C. albicans* (RCMB 05031). Ampicillin, gentamicin and Amphotericin B were used as reference drugs against Gram positive bacteria, Gram negative bacteria and fungi, respectively. DMSO alone was used as control at the same afore mentioned concentration and during this there was no visible change in bacterial growth. The plates were incubated at 37°C for 24 hrs for bacteria and for 48 hrs for fungi. The derivatives that showed significant growth inhibition zones using the twofold serial dilution technique, were further evaluated for their minimal inhibitory concentrations (MICs).



Characterization of the prepared CMCh derivatives

Minimal inhibitory concentration (MIC) measurement

The microdilution susceptibility test in Muller–Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) were used for the determination of antibacterial and antifungal activity, respectively. Stock solutions of the tested compounds, were prepared in DMSO. Each stock solution was diluted with standard method broth (Difco) to prepare serial twofold dilutions of the broth containing about 106 CFU/mL of test bacteria was added to each well of 96-well microtiter plate. The sealed microplates were incubated at 37°C for 24 hrs for antibacterial activity and at 37°C for 48 hrs for antifungal activity in a humid chamber. At the end of the incubation period, the minimal inhibitory concentrations (MICs) values were recorded as the lowest concentrations of the substance that had no visible turbidity. Control experiments with DMSO and uninoculated media were run parallel to the test compounds under the same conditions.

3. Results and Discussion

FTIR and H^1-NMRCharacterization of Schiff bases and their reduced derivatives

FTIR spectrum of chitosan showed four strong peaks at 1155, 1073, 1030, and 895 cm-1 which are characteristic peaks of the saccharide structure. The very strong broad peak around 3600-3200 cm-1 should be assigned to the stretching vibration of the O-H groups, the extension vibration of the N-H, and the intermolecular hydrogen bonds of the polysaccharide.

Primary amines have two peaks in this region. There are two weak absorption peaks at 1653 and 1567 cm-1 corresponded to amide I and amide II, respectively, which indicate that chitosan had a high degree of deacetylation. The FTIR spectrum of CMCh showed in addition to the aforementioned peaks, a strong peak at 1454 cm-1 which could be assigned to the symmetrical stretching vibration of COO- group. The asymmetrical vibration of COO- group (around 1550 cm-1) is overlapped with the deforming vibration of NH2 at 1604 cm-1 to obtain a very strong peak. The C-O absorption peak of hydroxyl group became stronger and shifted to 1097 cm-1. The obtained results, which are in accordance with the work of (Xie *et al.,* 2002a, b), indicated that substitution occurred at C6 position (Figure 1 (a)).

The FTIR data of the Schiff bases were listed in Table 1. (representative examples showed in Figure 1 (b-c))We can notice that the band between 3863-3167 cm-1 due to the O-H group stretching vibration is observed. In addition the characteristic absorbance of NH2 disappeared; these results indicate that –NH2 group had reacted with benzaldehyde derivatives. This is also well illustrated by the disappearance of the doublet peak at 3382 cm-1 and 3170 cm-1 corresponding to –NH2 group. Further, new peaks around 1698-1600 and 1384-1465 cm-1 appeared in the spectra which are the characteristic bands for the imine group (N=CH) and aromatic ring (C=C), respectively. H1-NMR data of CMCh and representative examples of its schiff base derivatives are listed in Table 2. (representative examples showed in Figure 2) For CMCh the following peaks appeared: δ for 2H (NH2) at 2.08 ppm, δ for 2H (CH2) at C6 at 2.50 ppm and δ for 3H (CH2COOH) at 3.88 ppm. The prepared Schiff base derivatives show similar peaks to CMCh, together with the disappearance of the peak corresponding to 2H of NH2 group, and the appearance of a new peak around 6.61-8.31 ppm which corresponding to H at N=CH. This, as well as a new peak around 6.89-9.66 ppm is corresponding to H of aromatic ring. In addition, the peaks according to the H of the substituent groups on the aromatic ring are also observed: δ for 3H (4-CH3) at 2.34 ppm, δ for H (4-OH) at 9.76 ppm, δ for 6H (3,4-dimethoxy) at 2.51 ppm, δ for 14 H (CH2) and 3H (CH3) at 1.24 ppm and 2.15 ppm respectively, and δ for 6H (dimethyl) at 2.49 ppm.

The FTIR data of the reduced derivatives were listed in Table 3. (representative examples showed in Figure 3 (b-c))We observe that the reduced derivatives show similar spectra as their parent Schiff bases except the disappearance of the imine (N=CH) peak around 1698-1600 cm-1 and the appearance of two new peaks at 1462-1419 cm-1 and 1604-1500 cm-1 which corresponding to CH2 and NH, respectively, which confirm the reduction of the CMCh Schiff base derivatives and the conversion of −N=CH− to −HN−CH2−. On the other hand, H1-NMR data of the reduced derivatives are listed in Table 4. (representative examples showed in Figure 4) Representative examples of the reduced derivatives showed the disappearance of the peak corresponding to the H of the imine group and the appearance of a new peak around 3.83-4.48 ppm which belong to 3H for −HN−CH2−which confirm the reduction process.

Scanning Electron Microscopy observations

Scanning electron micrographs of CMCh and some representative examples of its Schiff base derivatives and their corresponding reduced derivatives are shown in Figure 5 (a-i). These photos clearly show that the smooth surface of CMCh (Figure 5a) are nearly covered with the benzaldehyde derivatives as there is a change in size and shape of CMCh particles. The surface morphology change from a derivative to another, also we can notice that the surface of the same derivative differ from the Schiff base from its reduced form as example: CMCh-4-hydroxyBenz in Schiff base form differ completely from that in reduced form as we can clearly see needles in the reduced form. Finally it is possible to conclude that reaction between CMCh and benzaldehyde derivatives has been occurred which results in a complete change in the surface morphology of the prepared materials.

Solubility of CMCh Schiff bases and their reduced derivatives

The solubility of the Schiff bases and their reduced derivatives are tested in different polar or non-polar solvents. All the prepared materials were found to be completely soluble in hot dimethyl sulfoxide (DMSO). Concerning the Schiff base derivatives all the samples were insoluble in acetic acid except CMCh-2,4-dichloroBenz which is soluble. They are totally insoluble in water, ethanol, methanol, DMF, and chloroform. On the other hand, some of reduced derivatives were found to be soluble in acetic acid on cold like CMCh-4-methoxyBenz, CMCh-3-chloroBenz, CMCh-3,4-dimethoxyBenz, CMCh-2-thiophenecarboxyald., CMCh-3-bromoBenz, and CMCh-3-methoxyBenz, and some were found to be dissolved only on hot acetic acid like CMCh-Benz, CMCh-4-methylBenz, CMCh-4-chloroBenz, CMCh-4-bromoBenz, CMCh-2-chloroBenz, CMCh-2,4-dichloroBenz, and CMCh-2-methoxyBenz, where as some are insoluble like CMCh-4-hydroxyBenz, CMCh-4-nitroBenz, and CMCh-octaldehyde. All derivatives are totally insoluble in water, ethanol, methanol, DMF, and chloroform.

Antimicrobial activity

The antimicrobial activity of eighteenth of both Schiff bases of CMCh and their reduced derivatives were evaluated against *S. pneumoniae* (RCMB 010010) and *B. subtilis* (RCMB 010067) as Gram positive

bacteria, and against *E. coli* (RCMB 010052) as Gram negative bacteria and against *A. fumigatus* (RCMB 02568), *G. candidum* (RCMB 05097) and *C. albicans* (RCMB 05031) as fungi. Agar disk diffusion method was used for the determination of the preliminary antibacterial and antifungal activity. Ampicillin, gentamicin and Amphotericin B were used as reference drugs against Gram positive bacteria, Gram negative bacteria and fungi, respectively.

All of the synthesized substituted derivatives under investigation showed *in vitro* antimicrobial activity against the tested microorganisms.

The results of antibacterial activity of the CMCh, its Schiff bases and their reduced derivatives using inhibition zone method are listed in Tables (5-8). Compared with CMCh, its Schiff bases and their reduced forms have a higher antibacterial activity. Several mechanisms elucidating the antimicrobial activity of chitosan have been postulated. The most acceptable mechanism is the interaction between positively charged chitosan molecules and negatively charged microbial cell membrane. The interaction is mediated by the electrostatic forces between the protonated NH3+ groups of chitosan and the electronegative charges on the microbial cell surface. This electrostatic interaction results in twofold interferences: (1) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of the microorganisms, and (2) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions, and other low molecular weight proteinaceous constituents (e.g. protein, nucleic acid, glucose and lactate dehydrogenase) (Feng *et al.,* 2000). Since 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 CMCh with higher hydrophilicity, with better solubility in aqueous media and with greater positive density; where in CMCh 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 CMCh). 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 (Hadwiger *et al.,* 1986). Benzaldehyde derivatives grafted onto hydrophilic CMCh apart the CMCh chain away from each other, decrease their intermolecular hydrogen bonds, the reason for the easy of penetration of Schiff bases and their reduced forms 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 (Cuero *et al.,* 1991). It is established that both the –COOH and imine groups of CMCh Schiff bases and their reduced forms have excellent metal-binding capacity (Neveen *et al.,* 2011). This explain the observed higher antibacterial activity of Schiff bases and their reduced derivatives relative to CMCh.

CMCh Schiff bases and their reduced forms were more active against the Gram positive bacteria than against the Gram negative bacteria (Tables 5-8). As CMCh-4-nitroBenz Schiff base derivative caused inhibition zone diameter of *B. subtilis* and *S. pneumoniae* of 18.3, and 17 mm, respectively, corresponding to 14.6 mm of *E. coli* and also the inhibition zone diameter of the reduced form of the same derivative against *B. subtilis* and *S. pneumonia* and *E. coli* 19.5, 18.7 and 16.2 respectively (Table 5). This may be attributed to their different cell wall. The cell wall of Gram postitive bacteria is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of net works with plenty of pores, which allow foreign molecules to come into the cell without difficulty and allows more rapid absorption of ions into the cell. But the cell wall of Gram-negative bacteria is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the complicated bilayer cell structure, the outer membrane is a potential barrier against foreign molecules with high molecular weight. Therefore, the derivatives have different effects on the two kinds of bacteria. An additional evidence for the greater activity of CMCh Schiff bases and their reduced forms against Gram positive bacteria than that against Gram negative bacteria comes from their minimum inhibitory concentration (MIC) values. MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganisms after overnight incubation. The MIC values of CMCh-4-nitroBenz Schiff base derivative against *B. subtilis* and *S. pneumoniae* were 15.6 and 25 µg/ml, respectively, the MIC value against *E. coli* was 33 µg/ml and also MIC values of the reduced form of the same derivative against *B. subtilis* and *S. pneumonia*, and *E. coli* were found to be 12.5, 19 and 25 respectively (Table 5).

The results reveal that the antibacterial activity is not only affected by the nature of the substituent group found in the aryl ring of CMCh Scihff bases and their reduced forms but also by its position and its number in the mentioned ring.

A look at the results given in Table (5) directly reveals that these Schiff bases can be classified

according to their antibacterial activities into two main categories between which lies the non-substituted derivative. To the first category characterized by the greater antibacterial activity (greater inhibition zone diameter and lower MIC values) relative to that of the non-substituted derivative, belong all the Schiff bases and their reduced forms having electron-poor substituents or those decreasing the electron density at the imine group, thus increasing its cationic character. On the other hand, the Schiff bases and their reduced forms of lower antibacterial activity (smaller inhibition zone diameter and higher MIC values), relative to that of the non-substituted derivative, are characterized by the presence of either electron-rich substituents or those can donate electrons towards the imine group leading to decreasing its cationic character. The proof supporting this conclusion can be detected from the greater antibacterial activity of NO2 derivative relative to that of the bromo compound, which in its turn is greater than that of chloro derivative. This experimental proof is in good accordance with the greater electron withdrawing power of the nitro group relative to the bromo and the chloro groups.

Still another piece of evidence comes from the greater activity of the electron withdrawing group, as a substituent, in position 3 relative to its activity, if it is placed in positions 4 and 2 in the aryl ring. In the first case the electron withdrawing proceeds to a greater extent and much easier. On the other hand, the electron donating group, as a substituent, showed a greater activity if it is placed in positions 2 and 4 in the aryl ring relative to its activity at position 3. Table 6 shows the inhibition zone diameter values of chloro, bromo, and methoxy derivatives in which the substituent group occupies different positions on the aryl ring (ortho, meta, or para).

Moreover, Table 7 shows the inhibition zone diameter values for the derivatives which have more than one substituent group on the aryl ring such as CMCh-3,4-dimethoxyBenz and CMCh-2,4-dichloroBenz. In case of electron withdrawing derivatives the results clearly reveal that increasing the number of the substituent groups increase the antibacterial activity but still lower than that of the derivative which have the substituent group in the meta-position. On the other hand, in case of electron donating derivatives, increasing the number of the substituent groups increase the antibacterial activity higher than that of the derivative which have the substituent group in the meta-position.

The rest of the derivatives also show high bacterial activity as shown in Table 8. The aliphatic derivative which is (CMCh-octaldehyde) showed lower antibacterial activity than the aromatic derivatives as the aryl ring play an important role in the antibacterial activity. Lipophilicity, which correlates well with the bioactivity of chemicals, is a very important molecular descriptor and different lipophilic behavior of compounds plays an important role in their biological activity mechanisms. The n-octanal/water partition coefficient (log Pow) is widely used as a general measure of lipophilicity. Compounds with benzyl groups have relatively higher log Pow values and hence shows more lipophilic character as compared to the compounds with aliphatic groups (Hoey *et al.,* 1996). Thus, the aliphatic derivative does not penetrate into the bacteria as easily as the derivatives with benzyl group do. This behavior can be attributed to the lower lipophilicity of the aliphatic derivatives.

Antifungal activity

The antifungal activities of CMCh Schiff bases and their reduced forms against *A. fumigatus* (RCMB 02568), *G. candidum* (RCMB 05097) and *C. albicans* (RCMB 05031) are shown in Tables (9-12). The results show that all the derivatives had effective activities against the tested fungi, compared with parent CMCh, with inhibitory indices ranging from 19.2 to 8.2 mm inhibition zone (Tables 9-12) and with MIC values ranging from 7.85 up to 555 µg/ml according to the type of the substituent of the derivative (Table 9).

Generally chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth (El-Ghaouth *et al.,* 1992). The antifungic mechanism of chitosan involves all 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 (Eweis *et al.,* 2006).

Again, the results also demonstrate how the antifungal activities are affected by the nature, position and number of the substituents in the aryl ring of the Schiff bases and their reduced forms. Thus, while the derivatives with groups of electron withdrawing nature show higher inhibition zone diameter and lower MIC values relative to that of those having electron donating nature, the unsubstituted derivative lies between these two extremes (Table 9).

Also, the greater antifungal activity is observed for electron withdrawing group, as a substituent, in position 3 relative to its activity if it is placed in position 2 and 4 in the aryl ring (Table 10). Also, increasing the number of the substituents increases the antifungal activity but still lower than that of the mono substituted derivatives in the meta position (Table 11). Finally, the aliphatic Derivtative show lower antifungal activity that the derivative having aryl ring (Table 12).

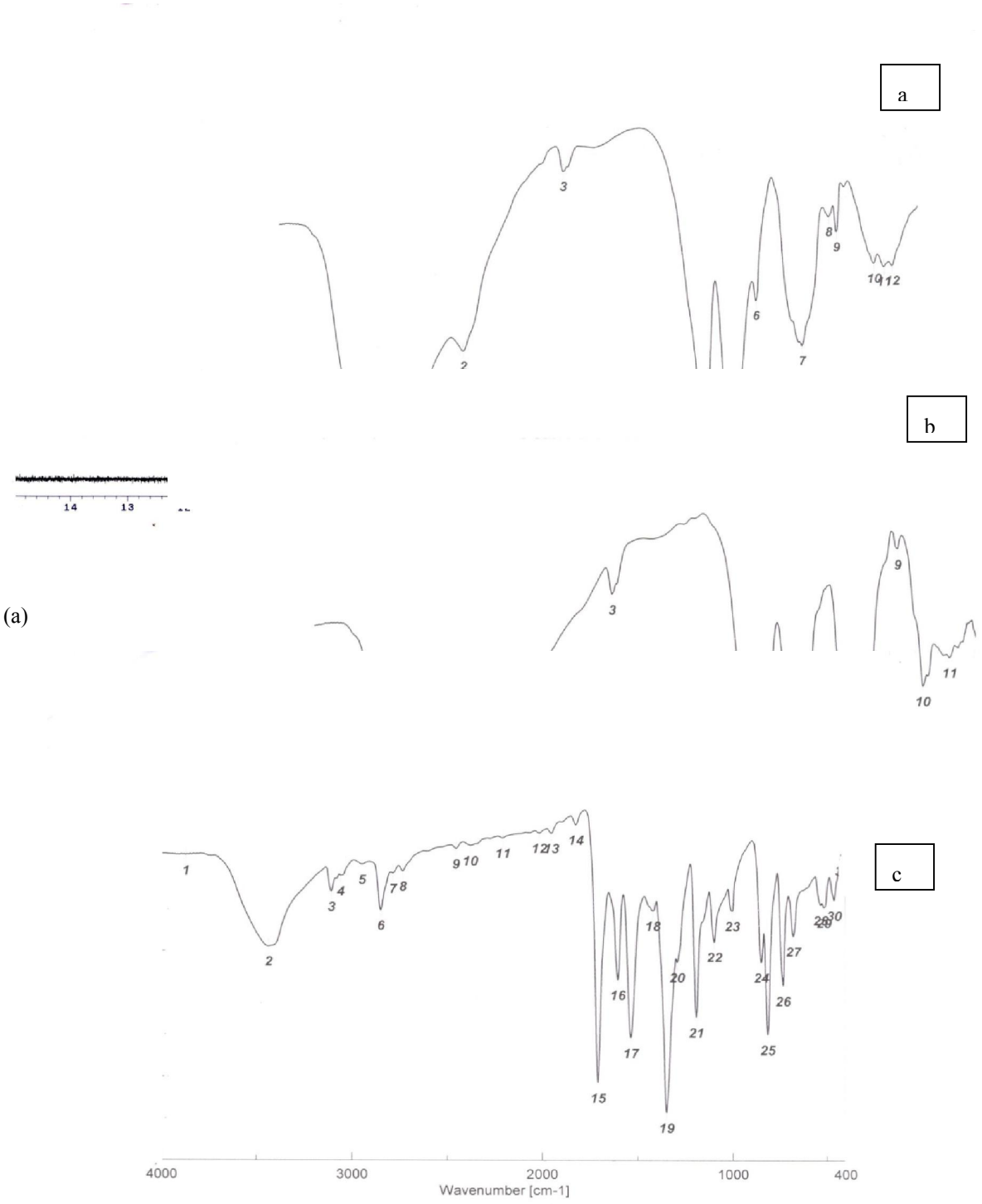


Figure 1. FTIR spectra of (a) CMCh, (b) CMCh-Benz Schiff base, and (c) CMCh-4-nitroBenz Schiff base.

(c)

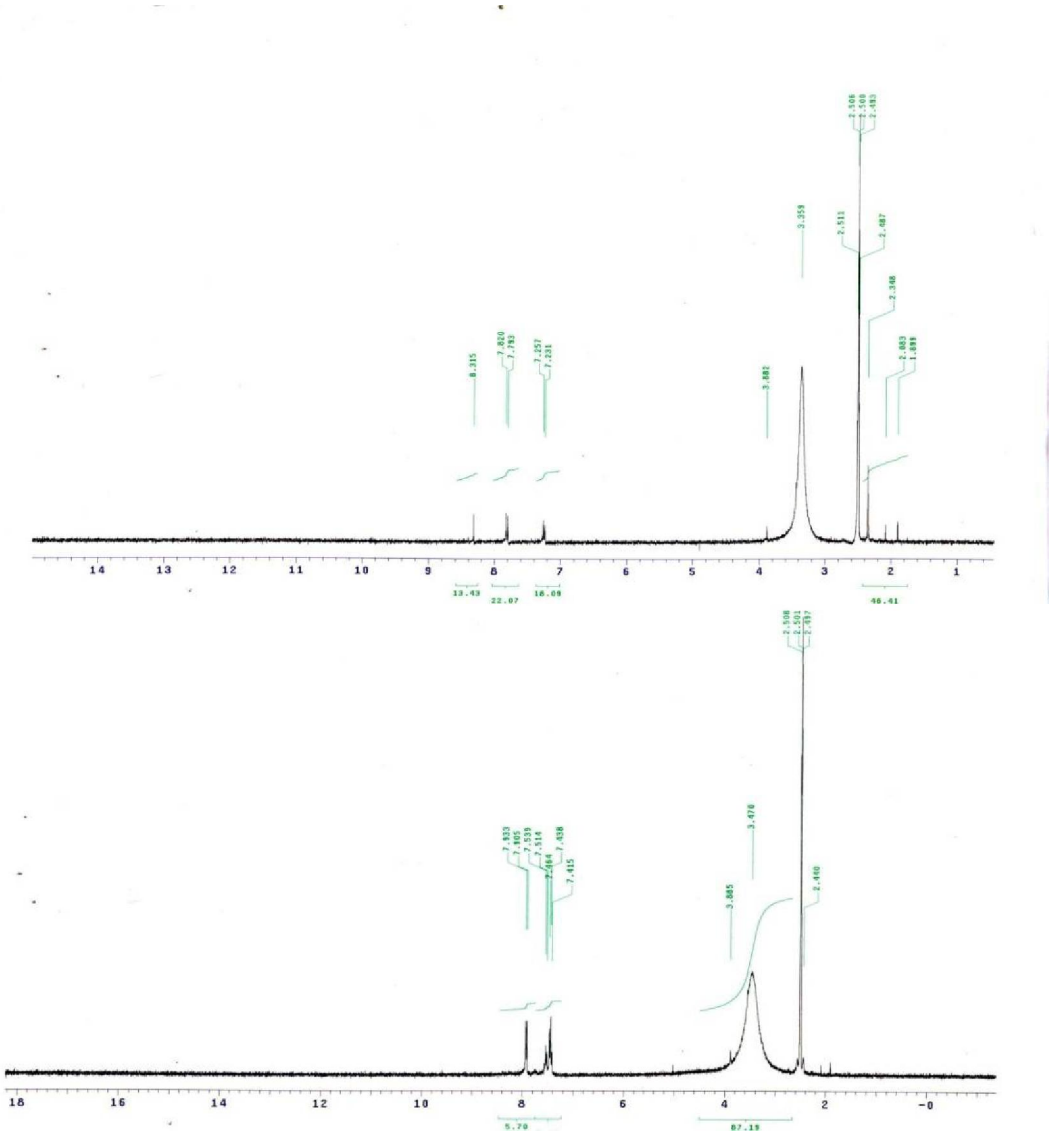
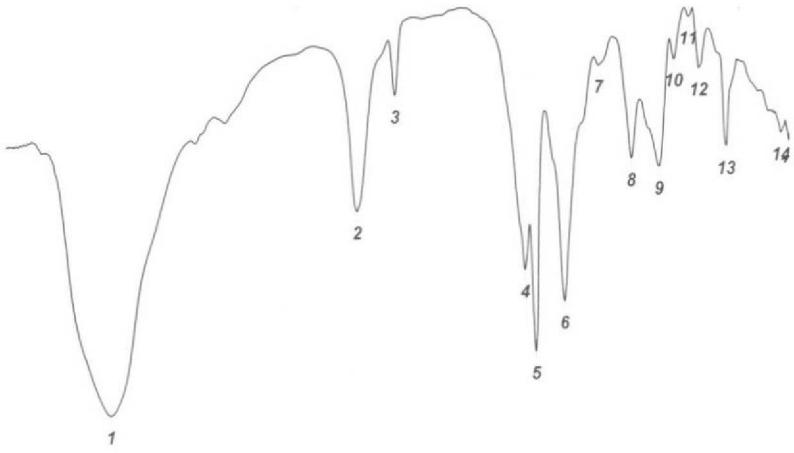


Figure 2. H[1]-NMR spectra of (a) CMCh, (b) CMCh-Benz Schiff base, and (c) CMCh-4-methylBenz Schiff base.

(a)

(b)



(c)

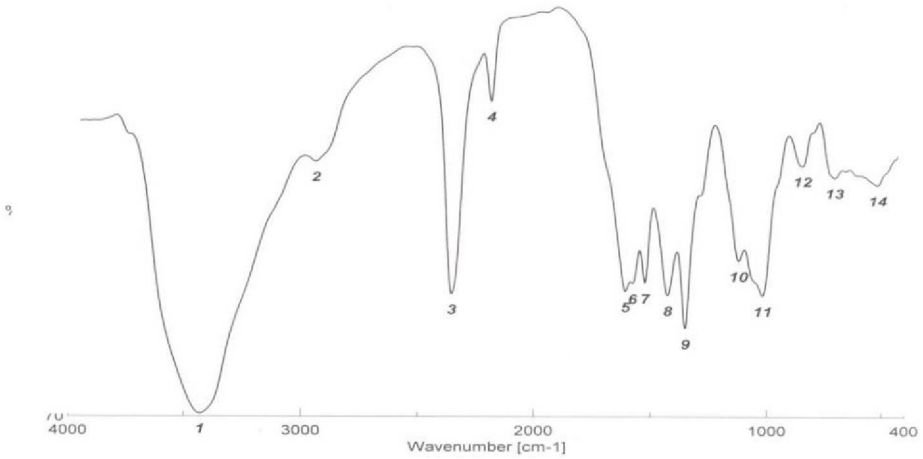


Figure 3. FTIR spectra of (a) CMCh, (b) CMCh-Benz reduced, and (c) CMCh-4-nitroBenz reduced.

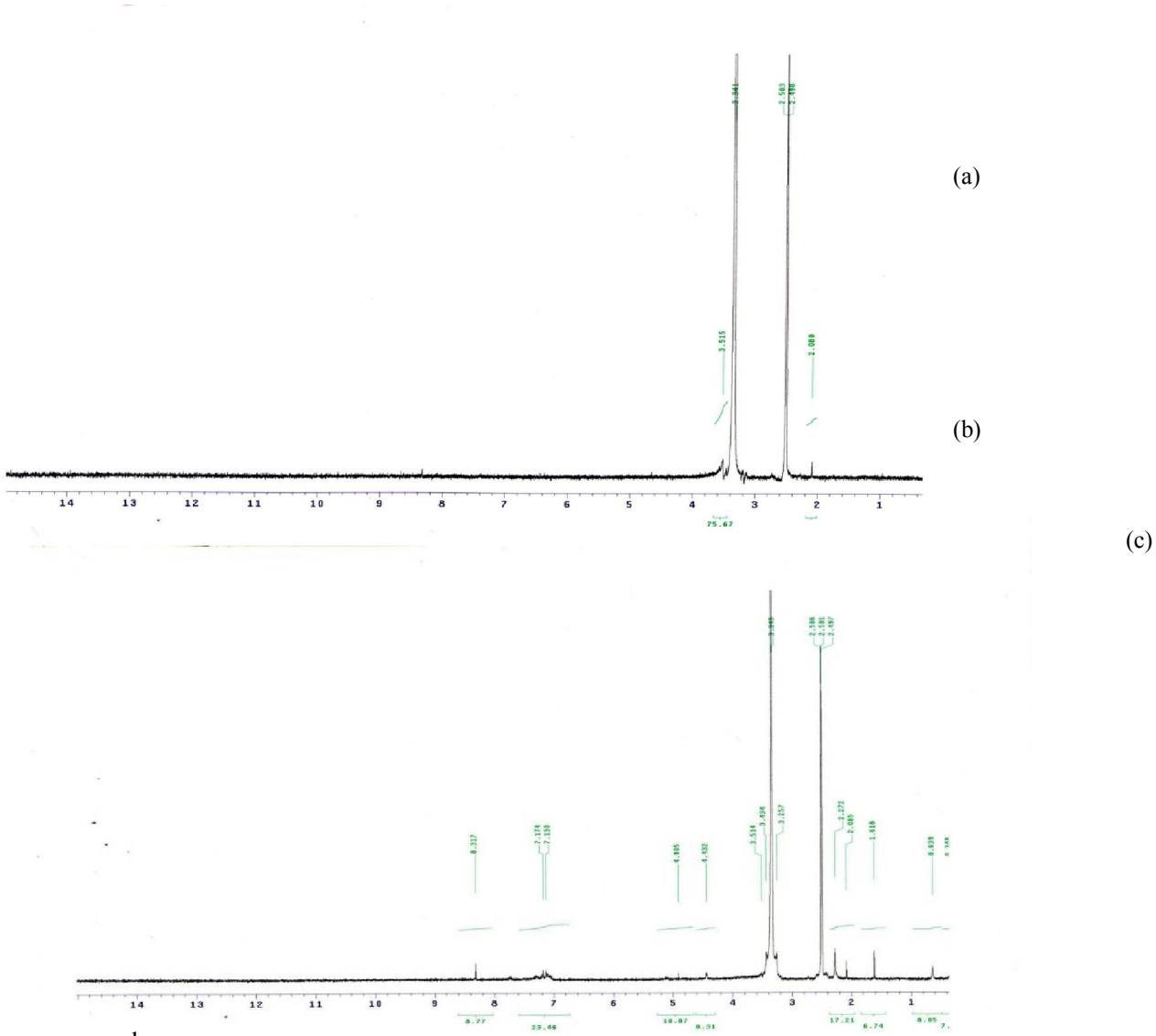
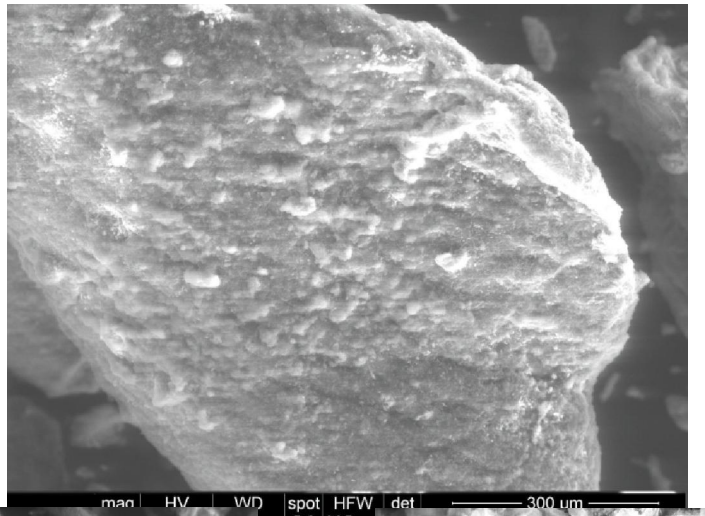
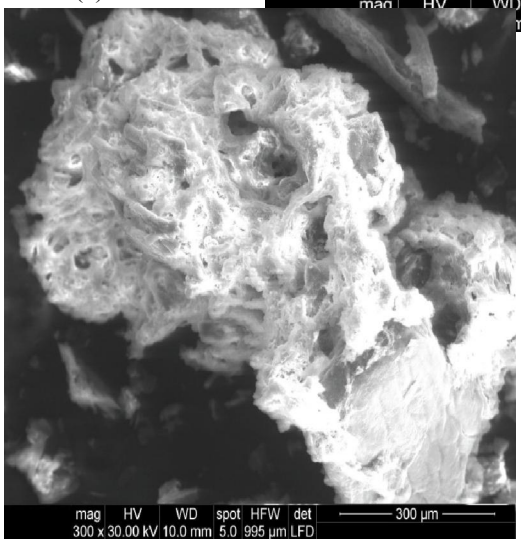
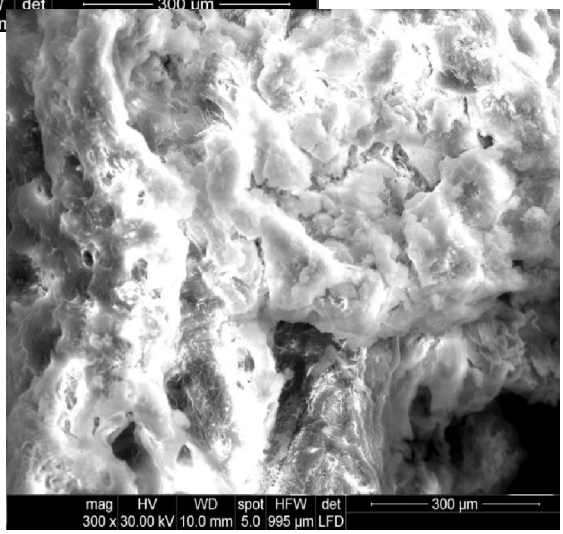


Figure 4. H[1]-NMR spectra of (a) CMCh, (b) CMCh-Benz Schiff reduced, and (c) CMCh-4-methylBenz Schiff reduced.

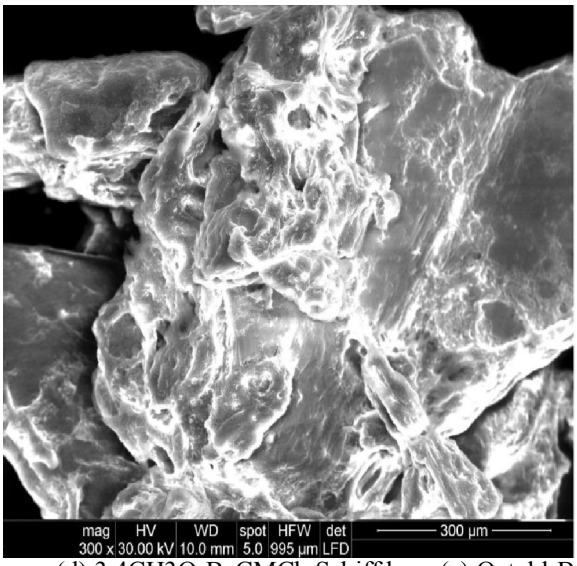


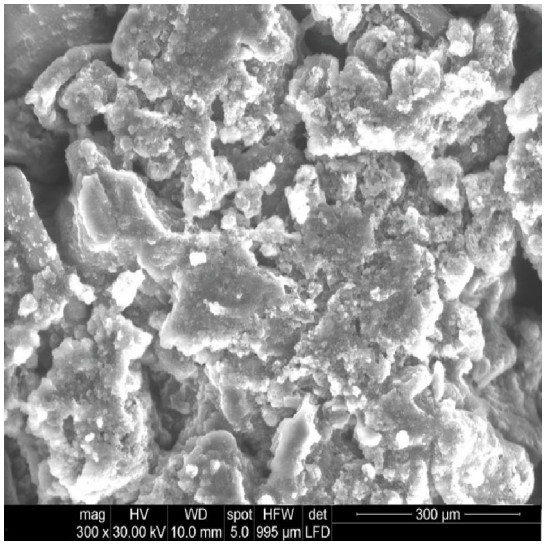
(a) CMCh.



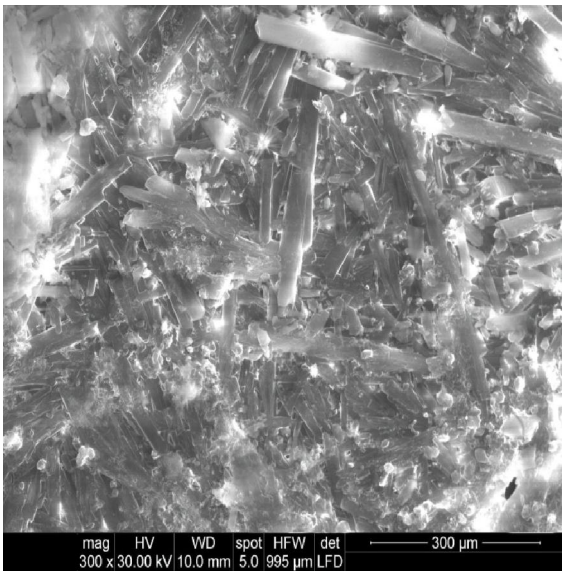


(b) 4HO-BzCMCh Schiff base. (c) 4CH3-BzCMCh Schiff base

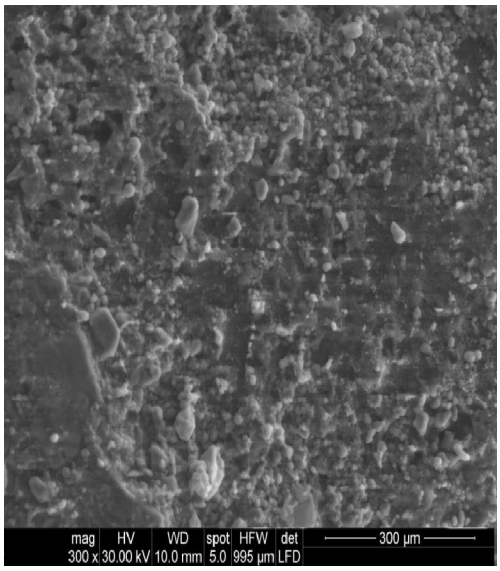




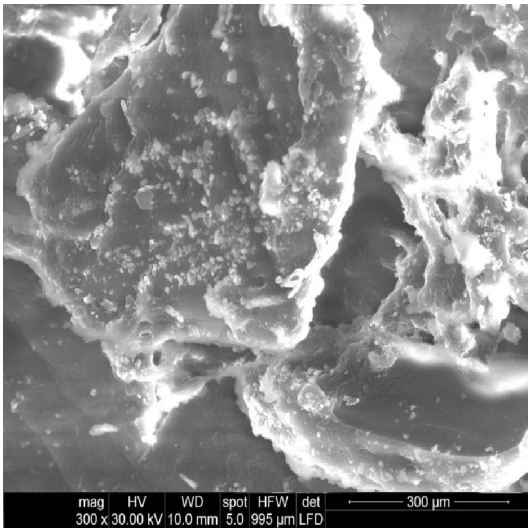
(d) 3,4CH3O-BzCMCh Schiff base (e) Octald-BzCMCh schiff base

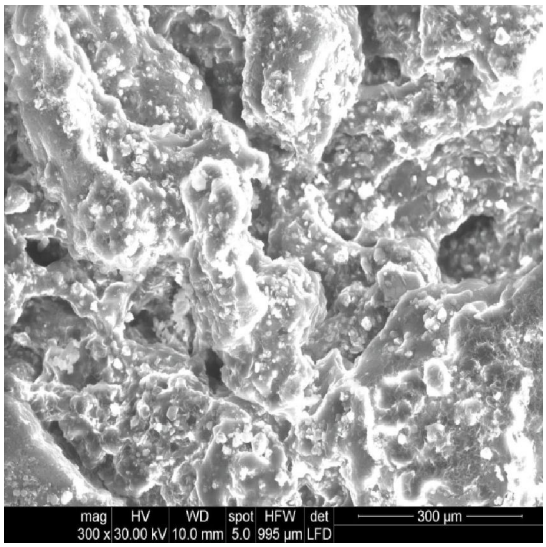


(f)



4HO-BzCMCh reduced. (g) 4CH3-BzCMCh reduced.





(h)

3,4CH3O-BzCMCh reduced. (i) Octald-BzCMCh reduced.

Figure 5. SEM photographs of CMCh Schiff bases and their reduced derivatives.

Table 1. FT-IR data of the prepared CMCh Schiff base derivatives

Type of vibration / Type of derivatives	O-H & N-H stretch	C-H stretch	C=N stretch	C-O	C-N	C-C Aromatic
CMCh-Benz	3441	2885	1600	1071	1319	1409
CMCh-4-methoxyBenz	3435	2926	1602	1034	1315	1425
CMCh-4-methylBenz	3437	2922	1600	1071	1310	1418
CMCh-4-nitroBenz	3863	2942	1603	1099	1348	1421
CMCh-4-bromoBenz	3430	2883	1600	1064	1317	1414
CMCh-4-hydroxyBenz	3167	2965	1671	1157	1388	1452
CMCh-4-chloroBenz	3429	2920	1600	1065	1316	1423
CMCh-3-chloroBenz	3445	2882	1697	1073	1304	1418
CMCh-3-bromoBenz	3429	2884	1641	1065	1380	1400
CMCh-3,4-dimethoxyBenz	3850	2932	1678	1016	1300	1457
CMCh-3-methoxyBenz	3733	2884	1698	1041	1320	1465
CMCh-2-chloroBenz	3837	2885	1632	1060	1319	1427
CMCh-2-methoxyBenz	3853	2923	1604	1060	1305	1405
CMCh-2,4-dichloroBenz	3430	2885	1632	1059	1384	1384
CMCh-2-thiophenecarboxyald.	3436	2882	1632	1061	1322	1425
CMCh-vanillin	3732	2932	1600	1029	1300	1452
CMCh-octaldehyde	3854	2928	1698	1074	1300	0
CMCh-4-dimethylaminoBenz	3863	2908	1600	1065	1313	1438

Table 2. H^1-NMR spectra of CMCh and its Schiff base derivatives.

Type of Chemical Shift / Type of derivatives	CH_2 at C6	CH_2COOH	2H (NH_2)	H at N=C	H for Ph	H according to the derivative
CMCh	2.50 ppm	3.88 ppm	2.08 ppm	-	-	-
CMCh-Benz	2.50 ppm	3.88 ppm	-	8.00 ppm	5H 7.40-7.90 ppm	-
CMCh-4-methylBenz	2.50 ppm	3.35 ppm	-	8.31 ppm	4H 7.20-7.80 ppm	3 H (CH_3) 2.34 ppm
CMCh-4-hydroxyBenz	2.50 ppm	3.43 ppm	-	7.72 ppm	4H 6.89-7.70 ppm	H (OH) 9.76 ppm
CMCh-3,4-dimethoxyBenz	2.50 ppm	3.34 ppm	-	7.58 ppm	3H 7.16-7.57 ppm	6H (OCH_3) 2.51
CMCh-octaldehyde	2.50 ppm	3.35 ppm	-	6.61 ppm	-	14H (CH_2) 1.24 ppm & 3H (CH_3) 2.15 ppm
CMCh-4-dimethylaminoBenz	2.50 ppm	3.33 ppm	-	7.69 ppm	6.77-9.66 ppm	6H (2 CH_3)2.49 ppm

Table 3. FT-IR data of reduced derivatives of CMCh Schiff bases.

Type of vibration / Type of derivatives	O-H & N-H stretching	C-H stretch	-COO- stretch	C-O	$-CH_2-$ bend	N-H bend
CMCh-Benz	3437	2348	1605	1010	1432	1560
CMCh-4-methoxyBenz	3852	2934	1613	1128	1458	1510
CMCh-4-methylBenz	3450	2351	1625	1006	1424	1590
CMCh-4-nitroBenz	3430	2932	1603	1014	1423	1572
CMCh-4-bromoBenz	3444	2935	1624	1065	1419	1500
CMCh-4-hydroxyBenz	3864	2960	1604	1010	1457	1513
CMCh-4-chloroBenz	3852	2352	1620	1013	1420	1590
CMCh-3-chloroBenz	3426	2984	1696	1073	1419	1572
CMCh-3-bromoBenz	3853	2872	1601	1022	1424	1593
CMCh-3,4-dimethoxyBenz	3853	2934	1607	1020	1458	1516
CMCh-3-methoxyBenz	3429	2933	1608	1033	1438	1500
CMCh-2-chloroBenz	3837	2355	1620	1007	1427	1604
CMCh-2-methoxyBenz	3853	2936	1680	1032	1458	1603
CMCh-2,4-dichloroBenz	3433	2348	1617	1006	1422	1500
CMCh-2-thiophenecarboxyald.	3429	2927	1625	1075	1427	1500
CMCh-vanillin	3374	2942	1600	1004	1458	1500
CMCh-octaldehyde	3844	2926	1644	1007	1462	1566
CMCh-4-dimethylaminoBenz	3853	2910	1661	1062	1439	1598

Table 4. H^1-NMR spectra of reduced derivatives of CMCh Schiff bases.

Type of Chemical Shift / Type of derivatives	CH_2 at C6	CH_2COOH	$2H(NH_2)$	3 H at $HN=CH_2$	H for Ph	H according to the derivative
CMCh	2.50 ppm	3.88 ppm	2.08 ppm	-	-	-
CMCh-Benz	2.50 ppm	3.34 ppm	-	4.48 ppm	5H 7.24-7.85 ppm	-
CMCh-4-methylBenz	2.50 ppm	4.90 ppm	-	4.43 ppm	4H 7.13-8.31 ppm	3 H (CH_3) 2.27 ppm
CMCh-4-hydroxyBenz	2.50 ppm	4.94 ppm	-	4.34 ppm	4H 6.63-7.10 ppm	H (OH) 9.76 ppm
CMCh-3,4-dimethoxyBenz	2.50 ppm	3.92 ppm	-	4.40 ppm	3H 6.80-8.32 ppm	6H (OCH_3) 2.50
CMCh-octaldehyde	2.50 ppm	3.35 ppm	-	3.83 ppm	-	14H (CH_2) 1.24 ppm & 3H (CH_3) 2.15 ppm
CMCh-4-dimethylaminoBenz	2.50 ppm	4.91 ppm	-	4.34 ppm	6.65-7.69 ppm	6H (2 CH_3)2.85 ppm

Table 5. Effect of the nature of the substituent groups of CMCh schiff bases and their reduced forms on their Antibacterial activity

Samples	Tested organisms					
	Gram positive bacteria				Gram negative bacteria	
	B. subtilis (RCMB 010067)		***S. pneumoniae*** (RCMB 010010)		***E. coli*** (RCMB 010052)	
	Inhibition zone(mm)	Minimum inhibitory concentration (MIC) (µg/ml)	Inhibition zone(mm)	Minimum inhibitory concentration (MIC) (µg/ml)	Inhibition zone (mm)	Minimum inhibitory concentration (MIC) (µg/ml)
CMCh	11.3	520	10.1	525	8.2	527
CMCh-4-nitroBenz	18.3 (19.5)	15.6 (12.5)	17.0 (18.7)	25 (19)	14.6 (16.2)	33 (25)
CMCh-4-bromoBenz	16.3 (18.3)	27 (20)	15.1 (16.3)	37 (27)	14.0 (15.0)	45 (35)
CMCh-4-chloroBenz	14.6 (16.3)	35 (29)	14.3 (14.9)	43 (37)	13.1 (14.0)	63 (45)
CMCh-Benz	13.5 (14.6)	47 (38)	13.1 (14.3)	79 (60)	12.3 (12.9)	116 (100)
CMCh-4-hydroxyBenz	12.4 (13.2)	167 (150)	12.5 (13.1)	183 (150)	-	-
CMCh-4-methoxyBenz	11.9 (12.6)	201 (178)	12.0 (12.6)	252 (200)	11.6 (12.0)	300 (205)
CMCh-4-methylBenz	11.6 (11.9)	500 (327)	10.5 (11.1)	500 (350)	8.6 (9.3)	500 (320)

Table 6. Effect of sub stituent position of CMCh Schiff bases and their antibacterial activity.
(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Samples	Tested micro-organisms		
	Gram positive bacteria		Gram negative bacteria
	B. subtilis (RCMB 010067) Inhibition zone(mm)	***S. pneumoniae*** (RCMB 010010) Inhibition zone(mm)	***E. coli*** (RCMB 010052) Inhibition zone(mm)
CMCh-2-chloroBenz	15.6 (17.3)	-	-
CMCh-3-chloroBenz	17.2 (19.1)	15.1 (17.2)	14.0 (14.6)
CMCh-4-chloroBenz	14.6 (16.3)	14.3 (14.9)	13.1 (14.0)
CMCh-2-methoxyBenz	14.3 (16.2)	13.3 (15.0)	12.2 (13.6)
CMCh-3-methoxyBenz	8.4 (9.7)	10.1 (12.0)	9.2 (11.0)
CMCh-4-methoxyBenz	11.9 (12.6)	12.0 (12.6)	11.6 (12.0)
CMCh-3-bromoBenz	19.2 (21.00)	16.9 (18.3)	14.6 (15.7)
CMCh-4-bromoBenz	16.3 (18.3)	15.1 (16.3)	11.1 (15.2)

(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Table 7. Effect of the number of substituents of CMCh Schiff bases and their reduced forms on antimicrobial activity.

Samples	Tested micro-organisms		
	Gram positive bacteria		Gram negative bacteria
	B. subtilis (RCMB 010067) Inhibition zone(mm)	***S. pneumoniae*** (RCMB 010010) Inhibition zone(mm)	***E. coli*** (RCMB 010052) Inhibition zone(mm)
CMCh-2,4-dichloroBenz	16.4 (18.6)	12.3 (14.4)	9.8 (11.2)
CMCh-2-chloroBenz	15.6 (17.3)	-	-
CMCh-3-chloroBenz	17.2 (19.1)	15.1 (17.2)	14.0 (14.6)
CMCh-4-chloroBenz	14.6 (16.3)	14.3 (14.9)	13.1 (14.0)
CMCh-3,4-dimethoxyBenz	10.5 (11.4)	11.5 (12.2)	10.3 (11.5)
CMCh-2-methoxyBenz	14.3 (16.2)	13.3 (15.0)	12.2 (13.6)
CMCh-3-methoxyBenz	8.4 (9.7)	10.1 (12.0)	9.2 (11.0)
CMCh-4-methoxyBenz	11.9 (12.6)	12.0 (12.6)	11.6 (12.0)

(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Table 8. Antibacterial activity of different CMCh Schiff base and their reduced forms.

Samples	Tested micro-organisms		
	Gram positive bacteria		Gram negative bacteri
	B. subtilis (RCMB 010067) Inhibition zone(mm)	***S. pneumoniae*** (RCMB 010010) Inhibition zone(mm)	***E. coli*** (RCMB 01005 Inhibition zone(mm)
CMCh-4-dimethylaminoBenz	18.3 (20.3)	16.6 (18.3)	11.7 (13.3)
CMCh-2-thiophenecarboxyald.	17.2 (18.6)	13.8 (15.1)	10.2 (12.6)
CMCh-vanillin	15.0 (16.1)	13.7 (14.3)	10.0 (11.3)
CMCh-octaldehyde	14.2 (15.6)	11.8 (12.4)	9.2 (10.5)

(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Table 9. Effect of the nature of the substituent of CMCh schiff bases and their reduced forms on their antifungal activity

Samples	Tested organisms					
	Fungi					
	. Fumigatus (RCMB02568)		***G. candidum*** (RCMB 05097)		***C. albicans*** (RCMB 05031)	
	Inhibition zone(mm)	Minimum inhibitory concentration (MIC) (µg/ml)	Inhibition zone (mm)	Minimum inhibitory concentration (MIC) (µg/ml)	Inhibition zone(mm)	Minimum inhibitory concentration (MIC) (µg/ml)
CMCh	12.1	550	9.3	540	5.4	555
CMCh-4-nitroBenz	16.2 (17.5)	31.25 (25)	17.3 (19.5)	7.85 (6.5)	16.4 (17.4)	25 (20)
CMCh-4-bromoBenz	15.3 (16.3)	40.35 (32)	16.1 (16.8)	15.65 (10)	5.5 (16.2)	62.5 (55)
CMCh-4-chloroBenz	14.9 (15.1)	52.5 (43)	15.9 (16.3)	25 (15)	14.6 (15.2)	125 (100)
CMCh-Benz	14.1 (14.9)	79 (59)	14.1 (15.0)	62.5 (50)	14.0 (14.0)	125 (100)
CMCh-4-hydroxyBenz	-	125 (110)	13.4 (14.6)	125 (85)	13.4 (13.2)	200 (150)
CMCh-4-methoxyBenz	13.7 (14.2)	200 (142)	12.3 (14.1)	200 (100)	12.0 (12.3)	500 (200)
CMCh-4-methylBenz	12.5 (12.8)	500 (350)	9.6 (10.2)	500 (359)	8.2 (9.3)	500 (362)

(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Table 10. Effect of substituent position of CMCh Schiff bases and their antifungal activity.

Samples	Tested micro-organisms		
	Fungi		
	Fumigates (RCMB 02568) Inhibition zone(mm)	***G. candidum*** (RCMB 05097) Inhibition zone(mm)	***C. albicans*** (RCMB 05031) Inhibition zone(mm)
CMCh-2-chloroBenz	-	(13.5)	(12.2)
CMCh-3-chloroBenz	14.1 (16.3)	17 (19.1)	15.3 (17.5)
CMCh-4-chloroBenz	14.9 (15.1)	15.9 (16.3)	14.6 (15.2)
CMCh-2-methoxyBenz	16.8 (18.3)	14.4 (16.3)	14.8 (16.9)
CMCh-3-methoxyBenz	12.8 (13.1)	10.2 (14.4)	9.3 (10.3)
CMCh-4-methoxyBenz	13.7 (14.2)	12.3 (14.1)	12.0 (12.3)
CMCh-3-bromoBenz	18.4 (20.1)	17.6 (19.3)	15.9 (17.5)
CMCh-4-bromoBenz	15.3 (16.3)	14.1 (16.8)	12.4 (15.2)

(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Table 11. Effect of the number of substituent group of CMCh Schiff bases and their reduced forms on antifungal activity

Samples	Tested micro-organisms		
	Fungi		
	Fumigatus (RCMB 02568) Inhibition zone(mm)	***G. candidum*** (RCMB 05097) Inhibition zone(mm)	***C. albicans*** (RCMB 05031) Inhibition zone(mm)
CMCh-2,4-dichloroBenz	19.2 (20.1)	16.4 (18.1)	14.3 (16.3)
CMCh-2-chloroBenz	-	13.5)	(12.2)
CMCh-3-chloroBenz	14.1 (16.3)	17 (19.1)	15.3 (17.5)
CMCh-4-chloroBenz	14.9 (15.1)	15.9 (16.3)	14.6 (15.2)
CMCh-3,4-dimethoxyBenz	15.6 (17.2)	12.4 (13.9)	11.1 (12.1)
CMCh-2-methoxyBenz	16.8 (18.3)	14.4 (16.3)	14.8 (16.9)
CMCh-3-methoxyBenz	12.8 (13.1)	10.2 (14.4)	9.3 (10.3)
CMCh-4-methoxyBenz	13.7 (14.2)	12.3 (14.1)	12.0 (12.3)

.(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

Table 12. Antifungall activity of different CMCh Schiff base and their reduced forms.

Samples	Tested micro-organisms		
	Gram positive bacteria		Gram negative bacteria
	B. subtilis (RCMB 010067) Inhibition zone(mm)	***S. pneumoniae*** (RCMB 010010) Inhibition zone(mm)	***E. coli*** (RCMB 010052) Inhibition zone(mm)
CMCh-4-dimethylaminoBenz	13.4 (15.6)	13.6 (14.7)	11.4 (13.0)
CMCh-2-thiophenecarboxyald.	16.9 (18.3)	14.3 (16.5)	12.5 (14.0)
CMCh-vanillin	13.3 (15.3)	13.6 (15.3)	12.0 (13.8)
CMCh-octaldehyde	9.3 (12.6)	13.3 (14.0)	8.3 (10.5)

(Data given in parentheses correspond to the reduced forms of CMCh Schiff bases)

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