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Preparation and antimicrobial activity of some chitosan-metal complexes against some plant pathogenic bacteria and fungi

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ABSTRACT

Four chitosan-metal complexes were prepared from low molecular weight chitosan with metals including Ag(I), Cu(II), Ni(II) and Hg(II) and were characterized by FT-IR. *In vitro* antimicrobial activities of the complexes were evaluated against the plant pathogenic bacteria of crown gall *Agrobacterium tumefaciens* and soft mold *Erwinia carotovora* and fungi of leaf spots and blights *Alternaria alternata*, grey mold *Botrytis cinerea*, root rot *Fusarium oxysporum* and damping off *Pythium debaryanum*. The complexes enhanced the antibacterial activity and chitosan-Hg and chitosan-Ag were the most active compounds, both of which had MIC of 15 and 30 mg/L against *E. carotovora*, respectively and 20 and 45 mg/L against *A. tumefaciens*, respectively. In addition, chitoan-metal complexes showed stronger antifungal activities (EC_{50} ranged from 14.50 to 869.49 mg/L) than the chitosan itself (higher than 2000 mg/L). Among the complexes, chitosan-Ag and chitosan-Hg exerted significantly prominent antifungal activity.

Keywords: Chitosan; Chitosan-metal complexes; antibacterial activity, antifungal activity.

INTRODUCTION

Among the novel families of biological macromolecules, whose relevance is becoming increasingly evident, is chitosan. Chitosan is a natural polymer composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (No and Meyers, 1997). It is soluble in acidic conditions due to the free protonable amino groups present in the D-glucosamine units. Due to its natural origin from a biopolymer chitin, chitosan can not be defined as a unique chemical structure but as a family of polymers which present a high variability in its chemical and physical properties. Chitosan and its derivatives received

considerable attention due to their potential biological activities. So it is used in different fields such as food, biomedicine, biotechnology, agriculture and cosmetics, among others (Kumar, 2000 and Rabea *et al*., 2003). The antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, algae, and some bacteria (El-Ghaouth *et al*., 1992 and Badawy and Rabea, 2009). It has several advantages over other types of disinfectants because it possesses a higher antimicrobial activity, a broader spectrum of activity and a lower toxicity toward mammalian cells (Liu *et al*., 2001). Many attempts have been taken up to improve its antimicrobial activity such as structural modification, adjustment of molecular factors, and forming complexes with other antimicrobial materials (Muzzarelli and Tanfani, 1985; Wang *et al*., 2005 and Badawy, 2010).

The -OH and -NH_2 groups on the skeleton of chitosan are good ligands to coordinate with transition metal ions to get chitosan-metal complexes. Recent years, chitosan-metal complexes attracted great interests for their potential use in agriculture, medical industry and food industry (Wang *et al*., 2004; Mekahlia and Bouzid, 2009 and Higazy *et al*., 2010). It is well known that both chitosan and metals such as Ag(I), Cu(II), Ni(II) and Zn(II) have the properties of disinfection and bactericide (Wang *et al*., 2005 and Mekahlia and Bouzid, 2009). After chitosan binds to metal ions through nitrogen and or oxygen, the bindings are likely to leave some potential donor atoms free and these free donor atoms enhance the antimicrobial activity (Wang *et al*., 2004). So it stands a good chance that chitosan-metal complexes exhibit enhanced ability of antimicrobial, which will be very favourable to chitosan-metal complexes' applications in agriculture, medical industry and food industry (Wang *et al*., 2004 and Mekahlia and Bouzid, 2009).

Up to date, there are many reports on antimicrobial activity of chitosan-metal complexes whereas, no work in the literature describing the use of chitosan-metal complexes to control plant pathogenic bacteria and fungi. Therefore, the present study is to investigate the antimicrobial activities of some chitosan-metal complexes to explore the structure-biological activity correlation against the most economic plant pathogens. In this paper, four chitosan complexes with Ag(I), Cu(II), Ni(II) and Hg(II) were prepared, and their structures were analyzed through FT-IR. The antimicrobial activities against bacteria of crown gall (*Agrobacterium tumefaciens*) and soft mold (*Erwinia carotovora*) and fungi of leaf spots and blights (*Alternaria*

alternata), grey mold (*Botrytis cinerea*), root rot (*Fusarium oxysporum*) and damping off (*Pythium debaryanum*) were studied *in vitro*.

MATERIALS AND METHODS

Materials: Low molecular weight of acid-soluble chitosan (made from coarse ground crab with 89% degree of deacetylation) was purchased from Sigma-Aldrich Co. (USA). Acetone, acetic acid, potassium bromide (KBr), silver nitrate ($AgNO_3$), copper sulphate ($CuSO_4.5H_2O$), nickel chloride ($NiCl_2.6H_2O$) and mercuric chloride ($HgCl_2$) were purchased from Adwic El-Nasr Pharmaceuticals Chemical Co. (Cairo, Egypt). Potato Dextrose Agar (PDA), Nutrient Broth (NB) and Nutrient Agar (NA) media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK).

Tested microorganisms: Bacteria of crown gall *Agrobacterium tumefaciens* (Family: Rhizobiaceae; Class: Alpha Proteobacteria) and soft mold *Erwinia carotovora* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria) and fungi of leaf spots and blights *Alternaria alternata* (Family: Dematiaceae; Class: Deuteromycetes), grey mold *Botrytis cinerea* (Family: Moniliaceae; Class: Deuteromycetes), root rot *Fusarium oxysporum* (Family: Tuberculariaceae; Class: Deuteromycetes) and damping off *Pythium debaryanum* (Family: Pythiaceae; Class: Oomycetes) were provided by the Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt.

Preparation of chitosan-metal complexes: Chitosan-metal complexes were prepared according to the method described by Wang *et al*., (2004). Chitosan (10 mmol, 1.61 g calculated as glucosamine unit) was dissolved in 50 ml aqueous acetic acid (1%). An exact known concentration of $AgNO_3$, $CuSO_4.5H_2O$, $NiCl_2.6H_2O$ and $HgCl_2$ pre-dissolved in distilled water and was added to the chitosan solution (corresponding to a molar ratio of 1:1 compared with chitosan residue). After addition of the salt, the pH value was adjusted to 7.0 by adding 0.1 M $NH_3 \cdot H_2O$ solution. The mixture was refluxed at 80°C for 3h with stirring. After it was cooled to room temperature, the mixture was poured into 200 ml acetone. The resulting precipitate was obtained by a filtration. The product was repeatedly washed with acetone and then oven-dried overnight at 60°C giving the chitosan-metal complexes. FT-IR spectra of chitosan and chitosan-metal complexes were recorded with KBr discs in the range of 400 - 4000 cm^{-1} on RXIFT-IR Spectrometer.

***In vitro* antibacterial activity assay:** The NA dilution method was used to determine a Minimum Inhibitory Concentration (MIC) of chitosan compounds to *A. tumefaciens* and *E. carotovora*. Stock solutions of chitosan compounds were prepared in aqueous acetic acid (0.5%, v/v) and the pH was adjusted to 5.5-6.0 with 1M NaOH. Serial dilutions of each compound were added to autoclaved NB medium (pH 7.0) to give the final concentrations ranging from 5 to 3000 mg/L and were then poured into sterilized Petri dishes. The culture of each bacterium in NB medium was diluted by sterile distilled water to 10^5-10^6 colony forming units (CFU)/ml. A loop of each bacterium suspension was inoculated on the surface of NA medium (ten spots per plate). After inoculation, the plates were incubated at 37°C for 24h. Each concentration and control was tested in triplicate. The MIC was determined as the lowest concentration of the compound required to completely inhibit bacterial growth comparing with the control (Speciale *et al*., 2002).

***In vitro* antifungal activity assay:** The antifungal activity of chitosan compounds on mycelial growth of *A. alternata*, *B. cinerea*, *F. oxysporum* and *P. debrianum* was tested using a radial growth technique (El Ghaouth *et al*., 1992). The compounds were dissolved in 0.5%, (v/v) aqueous acetic acid and the pH was adjusted to 5.5-6.0 with 1M NaOH. Serial concentrations of 250, 500, 1000, 1500, 2000, 2500 and 3000 mg/L were, respectively, added to sterilized PDA medium immediately before pouring into the Petri dishes. Each treatment was tested in triplicate. Mycelial discs (5 mm) of each pure culture, taken from 8-day-old cultures on PDA plates, were transferred aseptically to the centre of the Petri dishes. The plates were incubated in the dark at 26°C using an ISCO Incubator. The colony growth diameter was measured when the fungal growth in the control had completely covered the Petri dishes. Inhibition percentage of mycelial growth was calculated as follows:

$$\text{Mycelial growth inhibition (\%)} = \left[\frac{(\text{DC} - \text{DT})}{\text{DC}}\right] \times 100$$

Where DC and DT are average diameters of fungal colonies of control and treatment, respectively.

Statistical analysis: Statistical analysis was provided using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The effective concentration that inhibits 50% of fungal growth (EC_{50}) with its

95% confidence limits was estimated by probit analysis according to Finney (1971).

RESULTS AND DISCUSSION

Characterization of chitosan-metal complexes: Chitosan was proved to have the best chelating properties among other natural polymers (Varma *et al*., 2004). Responsible for complex formation are amino groups of chitosan, in which nitrogen is a donor of electron pairs, although hydroxyl groups may also participate in sorption. The mechanism of combining these reactive groups with ions of metals is much differentiated and can depend on the ion type, pH and also on the main components of the solution. The complexes formation could be also described based on Lewis acid-base theory: metal ions acting as the acid are the acceptor of a pair of electrons given by chitosan, acting as the base.

The IR spectra of chitosan-metal complexes as shown in Figure 1 exhibit many alterations from that of unmodified chitosan. A first broad and poorly resolved band around 3400-3450 cm^{-1} corresponds to the contribution of O-H stretching (from intra- and intermolecular hydrogen bonds) and N-H stretching (Wang *et al*., 1999, 2000; Tang and Hon 2001). A lot of differences in the spectra before and after metal binding were also observed in the environment of amine and amide groups (amide I band: 1656 cm^{-1} in chitosan, Figure 1A compared to 1622-1644 cm^{-1} in chitosan-metal complexes, Figure 1B-E; amide II band: 1570 cm^{-1} in chitosan compared to 1546-1558 cm^{-1} in chitosan-metal complexes). This group affected or disappeared as shown in Spectra 1B-E that suggests the amine or the acetamide group at C2 involved in metal binding (Mekahlia and Bouzid 2009, Tang and Hon 2001). Moreover, the intensity of the band at 1414 cm^{-1} in chitosan molecule was substantially affected after metal binding especially in the case of chitosan-Ag complex (Figure 1B). This peak attributes to bending vibration of -OH, which indicating -OH take part in metal binding.

Chitosan was proved to have the best chelating properties among other natural polymers (Varma *et al*., 2004). Responsible for complex formation are amino groups in which nitrogen is a donor of electron pairs, although hydroxyl groups can also participate in sorption. The mechanism of combining these reactive groups with ions of metals is much differentiated and can depend on the ion type and pH of the solution. The chitosan-metal

complexes formation could be also described based on Lewis acid-base theory: metal ions acting as the acid are the acceptor of a pair of electrons given by chitosan acting as the base. Based on this information, the reasonable structure of chitosan-metal complexes is shown in Figure 2 according to the hypothesis of Wang *et al*., (2004). As shown, metal ion like a bridge connected one or more chains of chitosan through interacting with -OH and -NH_2 groups.

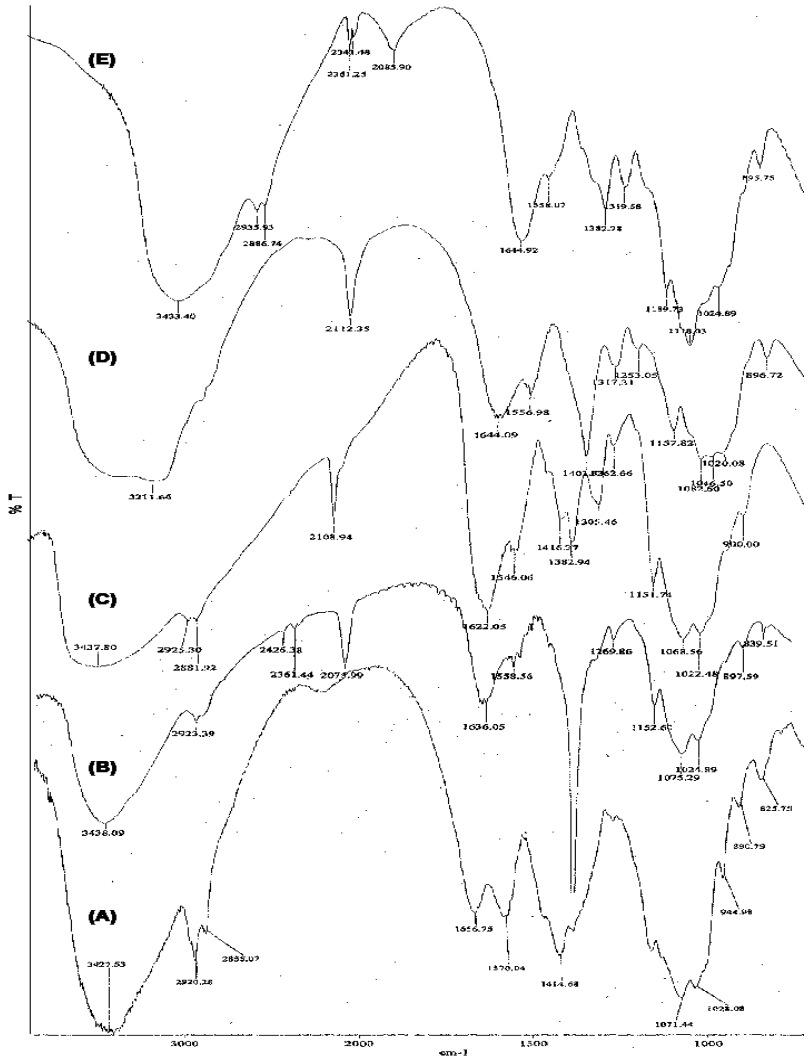

Figure 1. FT-IR spectra of chitosan and chitosan-metal complexes.
A: Chitosan (Ch 1), B: Ch 1-Ag, C: Ch 1-Hg, D: Ch 1-Ni and E: Ch 1-Cu.

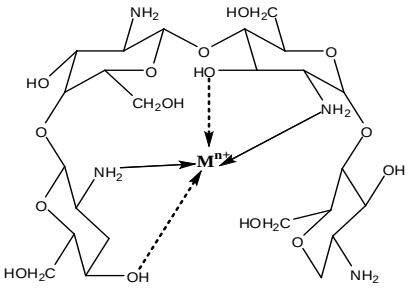

Figure 2. The reasonable structure of chitosan-metal complexes according to Wang *et al*. (2004).

Antibacterial activity of chitosan and chitosan-metal complexes: The minimum inhibitory concentration (MIC) values of chitosan and chitosan-metal complexes against bacteria of crown gall disease (*A. tumefaciens*) and soft mould disease (*E. carotovora*) are presented in Table 1. Generally, it was found that all tested chitosan-metal complexes were more potent than chitosan molecule, although differences existed among different kinds of metals and microorganism. Among all complexes tested, chitosan-Hg and chitosan-Ag were the most active compounds, both of which had MIC of 15 and 30 mg/L against *E. carotovora*, respectively and 20 and 45 mg/L against *A. tumefaciens*, respectively. When we consider the susceptibility of the microorganisms, another point deserves attention; the compounds under test had more effective inhibition on *E. carotovora* than *A. tumefaciens*. The activity of chitosan against these bacteria still low (MIC > 3000 mg/L) compared with that obtained in the literatures with other bacteria such as *Staphylococcus aureus* and *Escherichia coli* (Jia *et al*., 2001 and Goy *et al*., 2009). The fact may be attributed to the cell wall of *A. tumefaciens* and *E. carotovora*, which are typical Gram-negative bacteria. The cell wall of which is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules.

Table 1: *In vitro* antibacterial activity of chitosan and chitosan-metal complexes against *A. tumefaciens* and *E. carotovora*

Compound	MIC* (mg/L)	
	A. tumefaciens	*E. carotovora*
Chitosan	>3000	>3000
Chitosan-Ag	45	30
Chitosan-Cu	175	125
Chitosan-Ni	220	170
Chitosan-Hg	20	15

*Minimum Inhibitory Concentration

Chitosan is considered to be a bacteriocidal (kills the live bacteria or some fraction therein) or bacteriostatic (hinders the growth of bacteria but does not imply whether or not bacteria are killed), often with no distinction between activities. Recent data in literature has the tendency to characterize chitosan as bacteriostatic rather than bactericidal (Coma *et al*., 2002), although the exact mechanism is not fully understood and several other factors may contribute to the antibacterial action (Raafat *et al*., 2008). Chitosan seems to be unable to pass the outer membrane of bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules (Helander *et al*., 2001). Therefore, direct access to the intracellular parts of the cells by chitosan is unlikely. The antibacterial mechanisms of chitosan suggested to be: the positive charge of the amino group at C-2 resulted in a polycationic structure which can be expected to interacted with the predominantly anionic components (lipopolysaccharides, proteins) of the microorganisms' surface (Helander *et al*., 2001 and Goy *et al*., 2009). The interaction resulted in great alteration of the structure of outer membrane, which caused release of major proportion of proteinaceous material from the cells (Vaara and Vaara, 1983).

The reaction between chitosan and metal ions of Ag, Cu, Ni and Hg may be described according to the Lewis acid-base theory (Fred and Ralph, 1967). Metal ions, acting as acceptor of electrons, showed stronger activity than H^+. When chelated with metal ions, the positive charge on the amino group of chitosan was strengthened. As a result, the chitosan complexes were easier to interact with anionic components of cell surface, and exhibited higher inhibitory activities.

Wang *et al*., (2004) studied the antimicrobial activities of five complexes with different Zn content against four Gram-positive bacteria, five Gram-negative bacteria and two fungi. The complexes showed a wide spectrum of effective antimicrobial activities, which were 2-8 and 4-16 times higher than those of chitosan and zinc sulfate, respectively. Also, the complexes had better antibacterial activity than antifungal activity, and showed very good activity particularly against *E. coli* and *Corynebacteria*, both with a MIC value of 3.13 mg/L.

Antifungal activity of chitosan and chitosan-metal complexes: The *in vitro* antifungal activity of chitosan and chitosan-metal complexes against fungi of *A. alternata*, *B. cinerea*, *F. oxysporum* and *P. debaryanum* is shown in Table 2. The data are expressed as effective concentration that inhibits 50% of mycelial growth (EC_{50}) with the corresponding 95% confidence limits. Chitosan slightly inhibited mycelial growth of the tested fungi with EC_{50} values of 2806.52, 2983.69, 2329.15 and 2180.12 mg/L for *A. alternata*, *B. cinerea*, *F. oxysporum* and *P. debaryanum*, respectively. As can be seen in the result, all chitosan-metal complexes showed stronger antifungal activities (EC_{50} ranged from 14.50 to 869.49 mg/L) than the unmodified chitosan. The potential reason is higher charge densities of such derivatives than chitosan. Among the complexes, chitosan-Ag and chitosan-Hg exerted significantly prominent antifungal activity (EC_{50} = 67.82 and 56.64 mg/L, respectively for *A. alternata*; 174.82 and 106.22 mg/L, respectively for *B. cinerea*; 57.03 and 42.04 mg/L, respectively for *F. oxysporum* and 51.16 and 14.50 mg/L, respectively for *P. debaryanum*). In regard to the susceptibility of the tested fungi, it can be seen that *F. oxysporum* and *P. debaryanum* as soilborne fungi are more susceptible to chitosan compounds than *A. alternata* and *B. cinerea* as airborne fungi.

Mechanisms proposed for the antifungal activity of chitosan focused mainly on its effect on fungal cell wall and cell membrane. The interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents (Allan and Hadwiger, 1979). Another mechanism is that the positively charged chitosan interacts with cellular DNA of some fungi, which consequently inhibits the RNA and protein synthesis (Hadwiger *et al*., 1986). Therefore, several research groups have started to modify a chitosan molecule to produce high positively charged chitosan derivatives with a high antimicrobial activity (Muzzarelli and Tanfani, 1985;

Badawy *et al*., 2004; Rabea *et al*., 2006; Badawy and Rabea, 2009 and Badawy, 2010).

Table 2 : *In vitro* antifungal activity of chitosan and chitosan-metal complexes against *A. alternata*, *B. cinerea*; *F. oxysporum* and *P. debaryanum*

Compound	EC_{50} (mg/L)	95% confidence limits (mg/L)		Slope ± SE	Chi square (χ^2)
		Lower	Upper		
		A. alternata			
Chitosan	2806.52	2385.69	3440.90	1.77±0.21	0.97
Chitosan-Ag	67.82	30.36	104.12	1.82±0.18	6.43
Chitosan-Cu	358.17	231.20	638.26	2.19±0.19	10.81
Chitosan-Ni	360.19	186.75	786.38	3.01±0.24	23.77
Chitosan-Hg	56.64	14.43	99.63	1.27±0.15	5.46
		B. cinerea			
Chitosan	2983.69	2306.76	4391.69	1.09±0.18	1.58
Chitosan-Ag	174.82	49.42	425.77	1.99±0.16	25.53
Chitosan-Cu	869.49	666.16	1286.49	1.54±0.19	1.96
Chitosan-Ni	587.67	487.58	747.24	1.81±0.19	4.39
Chitosan-Hg	106.22	43.04	186.66	2.53±0.20	19.20
		F. oxysporum			
Chitosan	2329.15	1924.29	2937.23	1.39±0.19	1.04
Chitosan-Ag	57.03	38.45	75.37	1.34±0.16	3.16
Chitosan-Cu	301.18	174.46	628.59	1.67±0.16	10.69
Chitosan-Ni	322.02	214.27	520.08	2.28±0.19	9.85
Chitosan-Hg	42.04	31.97	49.78	3.26±0.52	1.09
		P. debaryanum			
Chitosan	2180.12	1760.91	2821.72	1.22±0.18	1.91
Chitosan-Ag	51.16	12.61	87.50	1.76±0.19	8.11
Chitosan-Cu	108.37	62.62	160.37	2.49±0.18	9.30
Chitosan-Ni	53.87	9.57	96.49	1.73±0.19	10.09
Chitosan-Hg	14.50	7.67	20.38	3.11±0.28	10.97

CONCLUSION

Chitosan-metal complexes with differed bivalent chelating metal ions of Ag, Cu, Ni and Hg were prepared and characterized with FT-IR. The quantitative antibacterial and antifungal effect of these compounds were evaluated against the bacteria of *A. tumefaciens* and *E. carotovora* and the fungi of *A. alternata*, *B. cinerea*, *F. oxysporum* and *P. debaryanum*. The tested chitosan complexes showed high antimicrobial activities against

tested microorganisms, which were much higher than free chitosan molecule. Through the discussion upon the antimicrobial mechanism of chitosan-metal complexes, it is concluded that the chitosan-metal complexes showed higher antimicrobial activities because of the stronger positive charge after complexation. It appeared that chitosan-metal derivatives at the applied concentrations exhibited a wide range of antibacterial and antifungal activity *in vitro*, affecting different plant pathogens.

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تحضير ونشاط ميكروبى لبعض معقدات الكيتوزان مع المعدن ضد بعض البكتيريا والفطريات الممرضة للنبات

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تتركز هذة الدراسة على تحضير أربعة معقدات من مركب الكيتوزان المنخفض الوزن الجزيئى مع معادن الفضة, النحاس, النيكل والزئبق وقد تم التأكد من التركيب الكيميائى لهذة المركبات بواسطة جهاز الأشعة تحت الحمراء FT-IR. بعد ذلك تم التقييم البيولوجى ضد البكتيريا المسببة لمرض التدرن التاجى Agrobacterium tumefaciens والبكتيريا المسببة للعفن الطرى لثمار الخضروات والفاكهه Erwinia carotovora و ضد كلا من فطر Alternaria alternata المسبب لتبقع الأوراق , فطر Botrytis cinerea المسبب للعفن الرمادى , فطر Fusarium oxysporum المسبب لعفن الجذور وسقوط البادرات وفطر Pythium debaryanum المسبب لمرض الذبول الطرى فى البادرات. أوضحت النتائج أن هذة المعقدات حسنت النشاط الإبادى البكتيرى وكان معقد الكيتوزان-زئبق ومعقد الكيتوزان-فضة أعلى المركبات تأثير حيث كانت قيمة التركيز المسبب لموت 100% من البكتيريا(MIC) هى 15 و 30 ملجم/لتر على التوالى ضد بكتيريا E. carotovora و 20 و 45 ملجم/لتر ضد بكتيريا A. tumefaciens على التوالى. بالإضافة إلى ذلك فقد أظهرت معقدات الكيتوزان مع المعادن نشاط إبادى فطرى قوى عن الكيتوزان (قيم التركيز المسبب لتثبيط 50% من النمو الفطرى تتراوح من 14.50 إلى 869.49 ملجم/لتر) ومن بين هذة المعقدات معقد الكيتوزان-فضة ومعقد الكيتوزان- زئبق حيث أعطىأعلى تأثير معنوى إبادى فطرى.