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ORIGINAL ARTICLE

Synthesis and antimicrobial activity of α-aminophosphonates containing chitosan moiety

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Abstract A novel series of α-aminophosphonates containing chitosan moiety was obtained in high yields from reactions of chitosan with aromatic aldehydes and triphenylphosphite in the presence of lithium perchlorate as a catalyst. The structures of the synthesized compounds were confirmed by IR and ^{1}H NMR spectral data. Compounds (**1**–**4**) showed high antimicrobial activities against *Escherichia coli* (NCIM2065), *Serratia marcescens*, *Enterobacter cloacae*, *Shigella dysenteriae*, *Salmonella enterica* and *Proteus vulgaris* as Gram-negative bacteria, *Bacillus subtilis* (PC1219) and *Staphylococcus aureus* (ATCC25292) as Gram-positive bacteria and *Candida albicans* as a fungus, at low concentrations (2.5–10 mg/mL).

1. Introduction

Chitosan is typically obtained by deacetylation of chitin under alkaline conditions, which is one of the most abundant organic materials; chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by (**1**–**4**) glycosidic bonds (Alves and Manoa, 2008). Chitosan displays interesting properties such as biocompatibility, biodegradability (Kumar et al., 2004; Sanford et al., 1989) and its degradation products are non-toxic, non-immunogenic and non-carcinogenic (Muzzarelli, 1997; Bersch et al., 1995). Chitosan is also used as a flocculant, clarifier, thickener, fibre, film and affinity chromatography column matrix gas-selective membrane, plant disease resistance promoter, anti-cancer agent, wound-healing promoting agent, and anti-microbial agent (Wang et al., 2002; Hu et al., 2002; Shin et al., 2001). Recently, there has been a growing interest in the chemical modification of chitosan in order to improve its solubility and widen its applications. Derivatization by introducing small functional groups to the chitosan structure, such as alkyl or carboxymethyl groups (Jayakumar et al., 1015; Lu et al., 2007) can drastically increase the solubility of chitosan at neutral and alkaline pH values without affecting its cationic character. Chemical modification of chitosan to generate new bifunctional materials is of prime interest because the modification would not change the fundamental skeleton of chitosan, would keep the original physicochemical and biochemical properties and finally would bring new properties depending on the nature of the group introduced. Recently, several methods have been reported to obtain phosphorylated derivatives

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of chitin and chitosan due to their interesting biological and chemical properties. They could exhibit bactericidal, biocompatible, bioabsorbable, osteoinductive and metal chelating properties (Jayakumar et al., 2005, 2008; Amaral et al., 2005; Wang et al., 2003; Fanny et al., 2005). Several techniques to obtain phosphorylated derivatives of chitin have been proposed. Phosphorylated chitin (Pchitin) and chitosan (P-chitosan) were prepared by the reaction of chitin or chitosan with orthophosphoric acid and urea in DMF (Hamodrakas et al., 1982a,b), with phosphorous pentoxide in methane sulphonic acid (Nishi et al., 1986), with phosphorous acid and formaldehyde (Ramos et al., 2003a,b), with diethyl chlorophosphate, NaOH, n-hexane (Cardenas et al., 2006), and with H_3PO_4/Et_3PO_4/P_2O_5/hexanol (Jayakumar and Tamura, 2006).

α-Aminophosphonates are among the most common organophosphorus derivatives and have been used as enzyme inhibitors, inhibitors of serine hydrolase, peptide mimics, antiviral, antibacterial, antifungal, anticancer, anti-HIV, antibiotics, herbicidal, and in other various applications (Li et al., 2007; Prasad et al., 2007; Rao et al., 2008; Naydenova et al., 2008, 2010; Tusek-Bozic et al., 2008; Wang et al., 2008; Rezaei et al., 2009; Onita et al., 2010; Zhang et al., 2010; Liu et al., 2010). Various methods for the synthesis of α-aminophosphonates were reported. However, one pot Mannich-type (Wang et al., 2001) process of carbonyl compounds, amines, and diphenyl phosphate in the presence of a Lewis acid catalyst remains the most efficient, simple, general, and high yielding method (El Sayed et al., 2011; Caldes et al., 2011; Ning et al., 2012; Reddy et al., 2012).

The present work was aimed to synthesize novel α-aminophosphonates containing chitosan moiety with the hope that new antimicrobial agents could be developed. Specifically, we grafted benzaldehyde, vanillin, *p*-chlorobenzaldehyde, cinnamaldehyde and triphenylphosphite in the presence of lithium perchlorate as a catalyst.

The biological activity of these modified chitosans was explored against *Escherichia coli* (NCIM2065), *Serratia marcescens*, *Enterobacter cloacae*, *Shigella dysenteriae*, *Salmonella enterica* and *Proteus vulgaris* as Gram-negative bacteria, *Bacillus subtilis* (PC1219) and *Staphylococcus aureus* (ATCC25292) as Gram-positive bacteria and *Candida albicans* as a fungus, at low concentrations (2.5–10 mg/mL).

To the best of our knowledge, no work on α-aminophosphonate conjugates with chitosan has been previously reported in the literature.

2. Experimental

2.1. Materials

Chitosan, benzaldehyde, *p*-chlorobenzaldehyde, cinnamaldehyde and vanillin were purchased from Aldrich.

2.2. Characterization techniques

IR spectra were recorded on a Perkin-Elmer 1430 Spectrophotometer using KBr disk technique. ^{1}H NMR spectra were recorded on a Bruker AC400 spectrometer operating at 400 MHz. The spectra were recorded in DMSO-d_6. Chemical shifts δ are reported in parts per million (ppm) relative to TMS. Assignments of signals are based on integration values and expected chemical shift values and have not been rigorously confirmed. Analytical thin layer chromatography (TLC) was performed on EM silica gel F_{254} sheet (0.2 mm) with chloroform/acetone (5:2 by volume) or petroleum ether (40–60 °C)/acetone (5:2 by volume) as developing eluents. The spots were detected with UV Lamp Model UV GL-58. Reagents and solvents were used from commercial sources without purification.

2.3. Synthesis of α-aminophosphonates containing chitosan moiety (*1*–*4*)

Chitosan (4.0 mmol) was added to a stirred mixture of aldehydes (2.0 mmol) and a solution of $LiClO_4$ in dichloromethane (DCM) (3.0 mL, 15.0 mmol; 5 M). The mixture was stirred at room temperature for 10 min before triphenylphosphite (0.93 g, 3.0 mmol) was added. The mixture was stirred at room temperature for 12–20 h before H_2O (10 mL) and DCM (10 mL) were added. The organic phase was separated and the aqueous layer was extracted with (DCM) (2×10 mL). The combined organics were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to give the crude product which was recrystallized from dimethyl sulphoxide (DMSO) to give pure product.

2.3.1. Characterization of compound *1*

IR (KBr): 3193, 1381, 900 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.93 (br s, exch., 1H, NH), 7.51–6.79 (m, Ar-H), 5.55 (d, J = 15 Hz, 1H, CH), 4.71–1.23 (m, carbohydrate protons).

2.3.2. Characterization of compound *2*

IR (KBr): 3404, 1382, 905 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.25 (br s, exch., 1H, NH), 7.40–6.73 (m, Ar-H), 3.84 (s, 3H, OCH_3), 9.77 (s, exch., 1H, OH), 5.55 (d, J = 14 Hz, 1H, CH), 4.70–1.23 (m, carbohydrate protons).

2.3.3. Characterization of compound *3*

IR (KBr): 3420, 1382, 900 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.28 (br s, exch., 1H, NH), 7.95–6.73 (m, Ar-H), 5.62 (d, J = 15 Hz, 1H, CH), 4.85–1.23 (m, carbohydrate protons).

2.3.4. Characterization of compound *4*

IR (KBr): 3445, 1383, 896 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.29 (br s, exch., 1H, NH), δ 8.13–6.72 (m, Ar-H), 5.60 (d, J = 17 Hz, 1H, CH), 4.66–1.23 (m, carbohydrate protons).

2.4. Antimicrobial activities

2.4.1. Test microorganisms

2.4.1.1. Gram-negative bacteria. After Gram-staining procedure, Gram-negative cells appear pink. The Gram-negative bacteria used in this study were *E. coli* (which is known as the back bone example for Gram-negative bacteria and cause urinary infection, wound infection and gastroenteritis), *Se. marcescens, En. cloacae, Sh. dysenteriae, Sa. enterica* and *P. vulgaris*.

2.4.1.2. Gram-positive bacteria. The thick cell wall of a Gram-positive organism retains the crystal violet dye used in the Gram-staining procedure, so the stained cells appear purple

under magnification. Gram-positive bacteria used in this study were *B. subtilis* and *St. aureus*. *B. subtilis* is mostly involved in Urinary infection, wound, ulceration and septicemia. *S. aureus* is the mile stone of Gram-positive bacteria and it is a causative agent of pneumonia, meningitis and food poisoning.

The tested bacteria were obtained from the culture collection of Bacteriology Unit, Department of Botany, Faculty of Science, Tanta University, Egypt.

2.4.1.3. Fungi. Pathogenic fungi spatially yeasts are responsible for a number of diseases in humans and animals. A number of pathogenic strains of fungi are represented in *C. albicans*.

The tested organisms were obtained from the culture collection of Mycology Unit, Department of Botany, Faculty of Science, Tanta University, Egypt.

2.4.2. Evaluation of antimicrobial activity

The antimicrobial spectrum of the prepared polymers was determined against the test bacteria on powdery samples by the cut plug method (Pridham et al., 1956) on nutrient agar which contained per litre: 10 g peptone, 5 g NaCl; 5 g beef extract and 20 g agar (pH 7). The assay plates were seeded with the test bacteria and fungi, after solidification the wells were made and filled with 20 mg powdery polymer. The plates were incubated at 30 °C for 24 h, after which the diameters of inhibition zones were measured and the compounds which produced inhibition zones were further assayed at different concentrations in aqueous suspension in order to quantify their inhibitory effects.

A loop full of each culture was placed in 10 ml of tenfold diluted broth, which was then incubated overnight at 30 °C. At this stage, the cultures of the test bacteria containing 6×10^4 cells/ml and the test fungus containing 8×10^2 spores/ml were used for the antimicrobial test. Since the polymers were not completely soluble either in water or in different solvents, they were suspended in a sterile ten times dilution of the above nutrient broth medium to make a 0.05 g/ml concentration and 0.5 ml was transferred to flasks containing sterile ten times diluted nutrient broth to give the final concentrations of 0.0, 2.5, 5.0 and 10.0 mg/ml. Exposure of bacterial cells to biocides was started when 0.2 ml of the culture containing 6×10^4 bacterial cells/ml or 8×10^2 fungal spores/ml was added to 10 ml of the above biocide suspension which was pre-equilibrated and shaken at 30 °C as recommended by Nakashima et al. (1987). At the same time, 0.2 ml of the same culture was added to 10 ml of tenfold diluted nutrient broth, decimal dilutions were prepared, and the starting number of cells was counted by spread plate method. After 24 h contact, 1.0 ml aliquots were removed and mixed with 9.0 ml of tenfold diluted nutrient broth and then decimal serial dilutions were prepared from these dilutions and the surviving bacteria or fungi were counted by the spread plate method. After inoculation, the plates were inoculated at 30 °C and the number of colonies was counted after 24 h for bacteria and 48 h for fungi. The ratio was carried out in triplicate every time. The ratio of the colony numbers for the media containing the polymers (*M*) and those without these compounds (*C*) was taken as surviving cell number and by this value the antimicrobial activity was evaluated.

2.4.3. Minimal inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) of the synthesized chitosan conjugate α-aminophosphonates (**1**–**4**) were determined for each antimicrobial activity against selected microorganisms by using agar diffusion method. The inhibition zone was measured in triplicates in four different concentrations (0.0, 2.5, 5.0 and 10.0 mg/mL) and the mean value ± standard deviation (SD) is recorded in Table 3 and Figs. 1–4.

3. Results and discussion

3.1. Chemistry

As part of continuing work in the area of biologically active compounds, we have recently reported the synthesis, antimicrobial, and anticancer activities of a novel series of α-aminophosphonates (Abdel-Megeed et al., 2012a,b,c,d, 2013).

Chitosan has an amino group at C-2 which is important because amino groups are nucleophilic and readily react with electrophilic reagents. In the present work, we exploited this reactivity for grafting biologically active moieties into the amino groups of chitosan to yield anti-microbial chitosans.

As a test case, the reaction of chitosan with benzaldehyde and triphenylphosphite in the presence of lithium perchlorate as a catalyst in dichloromethane (DCM) at room temperature was investigated. When chitosan (4 M equiv) was added a mixture of benzaldehyde (2 M equiv) and lithium perchlorate (15 M equiv) followed by the addition of triphenylphosphite (3 M equiv) in DCM at room temperature for 12 h, the yield of **1** was 85% (Table 1) after crystallization from dimethyl sulphoxide (DMSO). Consequently, the reactions of chitosan with various aldehydes (vanillin, *p*-chlorobenzaldehyde, cinnamaldehyde) and triphenylphosphite in the presence of lithium perchlorate as a catalyst were carried out in a similar condition.

The crude products were purified by crystallization from dimethyl sulphoxide (DMSO) to give α-aminophosphonates (**1**–**4**) Scheme 1 in high yields Table 1.

Table 1 indicates that the reaction represented in Scheme 1 is general, simple, high yielding, involves easy work-up and accommodates various substituents to produce various chitosan conjugate α-aminophosphonates. The structures of (**1**–**4**) were confirmed by IR and ^{1}H NMR spectroscopy.

The IR spectra of chitosan conjugate α-aminophosphonates (**1**–**4**) are characterized by the presence of absorption bands within the 3193–3445 cm^{-1} region corresponding to the stretching vibrations of the NH groups. While the absorption bands at the 1381–1383 cm^{-1} region are due to the symmetric stretching vibrations of the P═O groups and absorption bands within the 896–905 cm^{-1} region are attributed to the P–O–C groups.

The ^{1}H NMR spectra of chitosan conjugate α-aminophosphonates (**1**–**4**) showed a characteristic exchangeable singlet within the 9.29–7.93 ppm region due to NH proton. The CH protons resonated as doublet within the 5.62–5.55 (J = 14–17) ppm region and the protons of the carbohydrate moiety resonated at 4.85–1.23 ppm region.

Table 1 Synthesis of α-aminophosphonates containing chitosan moiety (**1–4**).

Yield (%)[b]	Aldehyde	Reaction time (h)[a]	Product
85	Benzaldehyde	12	**1**
81	Vanillin	15	**2**
75	*p*-Chlorobenzaldehyde	16	**3**
88	Cinnamaldehyde	13	**4**

[a] Completion of the reaction was tested by the use of TLC.
[b] Yield of pure products as colourless crystals after crystallization from ethanol.

Table 2 Antimicrobial activity (inhibition zones mm) of compounds (**1–4**).

Inhibition zone diameter [a] (mm)				Microorganisms
Compounds				
4	**3**	**2**	**1**	
26 ± 1.2	24 ± 1.2	28 ± 0.6	25 ± 0.0	*E. coli*
24 ± 1.2	20 ± 1.2	23 ± 1.0	23 ± 2.1	*S. marcescens*
24 ± 1.0	29 ± 1.7	26 ± 1.7	28 ± 2.1	*En. cloacae*
40 ± 0.6	26 ± 3.2	36 ± 1.2	35 ± 0.0	*Sh. dysenteriae*
32 ± 1.5	21 ± 1.0	30 ± 1.0	30 ± 0.0	*Sa. enterica*
27 ± 1.2	23 ± 0.6	26 ± 2.5	27 ± 2.4	*P. vulgaris*
27 ± 2.0	20 ± 0.0	27 ± 2.9	27 ± 2.2	*B. subtilis*
31 ± 1.0	21 ± 1.0	20 ± 1.0	14 ± 0.5	*St. aureus*
11 ± 0.0	12 ± 0.0	12 ± 0.6	13 ± 0.05	*C. albicans*

[a] DMSO was added to different organisms as control and showed no inhibition zone.

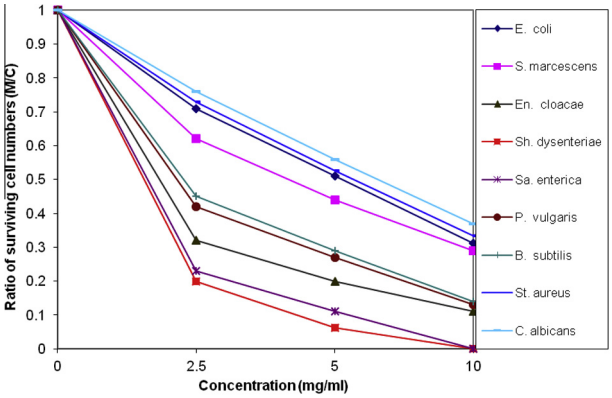


Figure 1 (MIC) of compound **1**.

3.2. Biology

3.2.1. Antimicrobial activities

The antimicrobial agents available on the market have various drawbacks such as toxicity, narrow spectrum of activity and some also exhibit drug–drug interactions. In view of the high incidence of infections in immune compromised patients, demands for new antimicrobial agents with a broad spectrum of activity and good pharmacokinetic properties have increased (Azizi and Saidi, 2003).

The synthesized chitosan conjugate α-aminophosphonates (**1–4**) were screened for their *in vitro* antibacterial and antifungal activities against *E. coli (NCIM2065)*, *S. marcescens*, *En. cloacae*, *Sh. dysenteriae*, *Sa. enterica* and *P. vulgaris* as Gram-negative bacteria, *B. subtilis (PC1219)* and *S. aureus (ATCC25292)* as Gram-positive bacteria and *C. albicans* as a fungus. The inhibition zones were measured in triplicates and the results of antimicrobial testing are reported in Table 2.

The results recorded in Table 2 showed that all compounds have high activities against bacteria and fungi. Compound **1** showed high inhibition zone against the Gram negative bacterium (*S. dysenteriae*) (35 mm). However, it showed relatively lower activities against the tested fungus (*C. albicans*) (13 mm). Compound 2 showed a similar trend as in case of compound 1 against the Gram negative bacterium (*S. dysenteriae*) (36 mm) and the tested fungus (*C. albicans*) (12 mm). Compound 4 showed high inhibition zone against the Gram

Table 3 Minimum inhibitory concentrations (MICs) for compounds (**1–4**).

Minimum inhibitory concentrations[a] (MICs) (mg/mL)				Microorganisms
Compounds				
4	**3**	**2**	**1**	
5.0 ± 0.1	5.0 ± 0.4	2.5 ± 0.4	5.0 ± 0.2	*E. coli*
10.0 ± 0.5	10.0 ± 0.4	5.0 ± 0.3	5.0 ± 0.3	*S. marcescens*
2.5 ± 0.4	2.5 ± 0.0	5.0 ± 0.7	10.0 ± 0.7	*En. cloacae*
5.0 ± 0.6	5.0 ± 0.3	2.5 ± 0.5	2.5 ± 0.2	*Sh. dysenteriae*
5.0 ± 0.2	5.0 ± 0.4	2.5 ± 0.2	2.5 ± 0.1	*Sa. enteric*
2.5 ± 0.4	2.5 ± 0.2	5.0 ± 0.4	5.0 ± 0.3	*P. vulgaris*
10.0 ± 0.3	10.0 ± 1.1	5.0 ± 0.6	5.0 ± 0.2	*B. subtilis*
5.0 ± 0.4	5.0 ± 0.4	10.0 ± 0.9	10.0 ± 0.4	*St. aureus*
20.0 ± 0.7	20.0 ± 1.4	20.0 ± 0.7	20.0 ± 0.3	*C. albicans*
7.2 ± 5.5	7.2 ± 5.5	6.4 ± 5.6	7.2 ± 5.5	Mean MICs (μg/mL)

[a] The standard antibiotic was Ampicillin (MIC = 5 μg/mL) and the standard antifungal was Itraconazole (50 mg/mL).

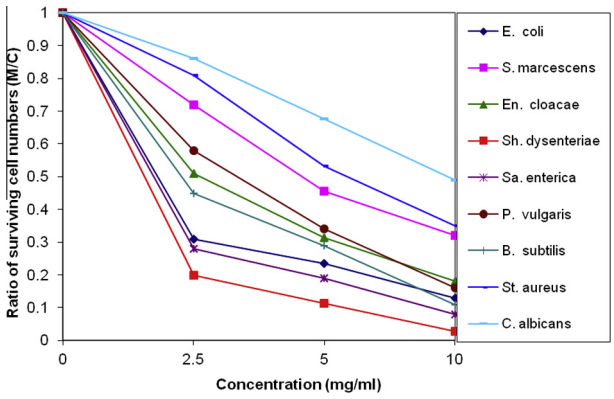


Figure 2 (MIC) of compound **2**.

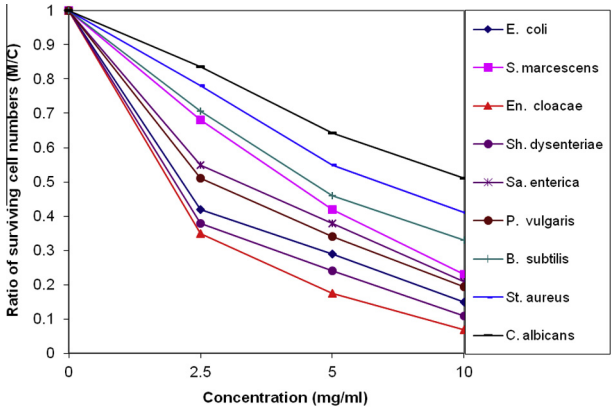


Figure 3 (MIC) of compound **3**.

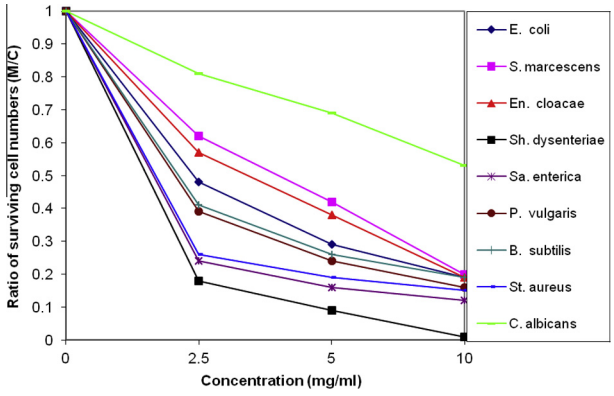
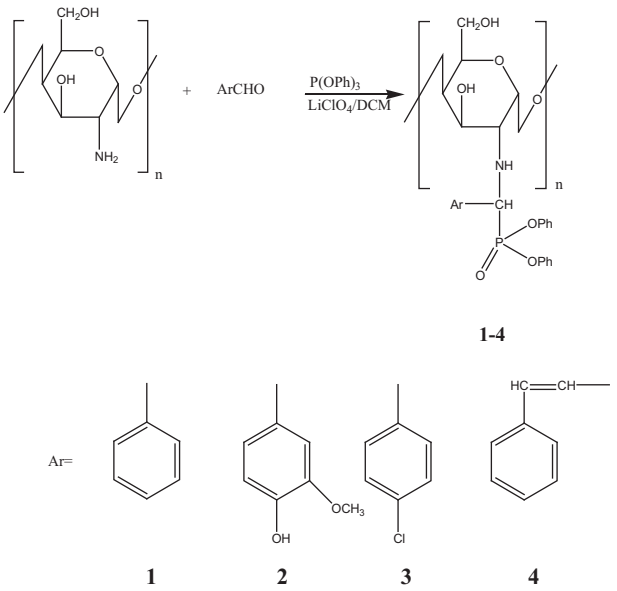


Figure 4 (MIC) of compound **4**.

negative bacterium (*S. dysenteriae*) (40 mm) and lower activities against the tested fungus (*C. albicans*) (11 mm).

3.2.2. Minimal inhibitory concentrations (MICs)

Table 3 revealed that all compounds showed high antimicrobial activities at low concentrations (2.5–10.0 mg/mL). Compound 1 showed MIC against *S. dysenteriae* and *S. enterica* lower than the tested microorganisms. The highest MIC was recorded against *C. albicans*. The results reported here look promising, since the activity against Gram negative bacteria is high. As it is well known that the structure of the Gram



Scheme 1

negative bacteria has extra cell wall layers which make extra-defense.

Compounds **1** and **2** showed higher activity against *S. dysenteriae*, they kill 80% and 90% respectively of the microorganism in the contact time at 2.5 mg/ml. Increasing the concentration from 2.5 to 10.0 mg/ml killed 100% of the species.

Compound **3** was more active against *E. cloacae* compared to the other tested microorganisms; it killed 95% at concentration 10 mg/ml.

Compound **4** was inhibitory to *S. dysenteriae* than the other test microorganisms. It killed 100% at concentration 10 mg/ml.

Generally, increasing the concentration from 2.5 to 10 mg/ml increased the activity against all tested microorganisms and the tested fungus was more resistant to the synthesized compounds than all bacterial strains.

4. Conclusion

A convenient method for the synthesis of modified chitosan was developed. The newly synthesized compounds exhibit remarkable antimicrobial activities against Gram-positive, Gram-negative bacteria and fungi at low concentrations.

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