



A comparative evaluation of antimicrobial activity of chitoooligosaccharides with broad spectrum antibiotics on growth of some pathogenic microorganisms

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ABSTRACT

The aim of this work was to determine the antimicrobial activity of chitoooligosaccharides (chito) and comparing them with that of the standards broad spectrum antibiotic flomox and kluacid. The chito were prepared by enzyme hydrolysis of chitosan. They were fractionated into four groups (1, 2, 3 and 4) according to their molecular weights (MW) by ultrafiltration. Specific growth rate of the tested microorganisms was determined in the presence of chito. The potential antimicrobial activity of chito was evaluated as minimum inhibitory concentration (MIC) towards Gram positive bacteria (*B. cereus*), Gram negative fungi (*P. aeruginosa*) and Gram positive yeast (*C. albicans*). MIC was determined by 8 serial dilution methods. The inhibitory activities of chito 1 and 3 were identified as being as strong as broad spectrum flomox and kluacid against *B. Cereus*. The specific growth rate of *C. albicans* was completely inhibited by chito (4) at concentration of 0.11 mg/ml with molecular weight less than 1.0 kDa and was stronger than that of broad spectrum kluacid (0.42 mg/ml). Chito (3) with molecular weight 1–10 kDa had MIC 1.67 mg/ml against *P. aeruginosa*. The results of this study suggested that chito can be a potent factor affecting as an antimicrobial activity. They could be considered as a possible alternative/additive to known antimicrobial agents in pharmaceutical compositions.

1. Introduction

Chitoooligosaccharides (chito) are the degraded products of chitosan by enzymatic or acidic hydrolysis. Enzymatic preparation methods have received great interest due to their safety and ease of control. Chito have attracted considerable interest due to their biological activities, namely, antimicrobial (Zhao and Xia, 2006; Selenius et al., 2018), hypocholesterolemic (Xia et al., 2011), immunity-enhancing and antitumor effects (Xia, 2003), drug delivery (Park et al., 2010) and accelerating calcium and ferrum absorption (Liao et al., 2007).

The antibacterial activity of chitoooligosaccharides is influenced by a number of factors such as degree of polymerization (Park et al., 2004a; Park et al., 2002), level of deacetylation (Chung et al., 2004), type of microorganism (Gerashenko et al., 2004; Park et al., 2004b) and some other physico-chemical properties.

Antibiotics are antimicrobial agents produced by microorganisms that inhibit the growth or kill other microorganisms while being harmless to the host cells. The determination of the susceptibility of

pathogens to antibiotics is necessary for the selection of the most appropriate one for treating microbial infections. Antibiotics which kill bacteria are said to be bactericidal, while those that only prevent their multiplication are referred to as bacteriostatic. However, some antibiotics can act as both bacteriostatic and bactericidal depending on their concentration. Antibiotics were evaluated for their inhibitory potentials. A few methods used for evaluating antibiotics include the filter paper disc (Kirby-Bauer) method (Bauer et al., 1966), and the dilution method (Wiegand et al., 2008; Owuama, 2015). The dilution method is mainly useful in determining minimum inhibitory concentration (MIC), which is the least concentration of antimicrobial agent that prevents microbial growth.

Growing number of diseases caused by microorganism not susceptible to antibiotics have increased problems with human allergy and have become a big problem globally (Montravers and Jabbour, 2006), besides that there are a limited number of antifungal drugs (Cowen et al., 2002). Therefore, there is a need for discover new natural antibiotics.

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In previous study, chitoooligosaccharides with different degrees of molecular weights were prepared previously through hydrolysis of colloidal chitosan by chitosanase (El-Sayed et al., 2016). In the present research, we investigated their antimicrobial activities (MIC) on selected strains namely, Gram positive bacteria (*Bacillus cereus*), Gram negative fungi (*Pseudomonas aeruginosa*) and Gram positive yeast (*Candida albicans*). These activities were compared with that of the standard antibiotic namely broad spectrum antibiotic flomox and kluacid.

2. Materials and methods

2.1. Chemicals

Chitosan with molecular weight 300,000 were purchased from Merck Chemical Co, Germany. Broad spectrum antibiotics, flomox and kluacid were used as standard drug. All chemicals were of analytical grade.

2.2. Tested microorganisms

Gram positive bacteria (*Bacillus cereus*), Gram negative fungi (*Pseudomonas aeruginosa*) and Gram positive yeast (*Candida albicans*) were obtained from MIRCIN culture collection of the faculty of Agriculture, Ain Shams University, Cairo, Egypt.

2.3. Preparation of chitoooligosaccharides

Chitoooligosaccharides were prepared by enzymatic hydrolysis of chitosan using immobilized pepper chitosanase (El-Sayed et al., 2016). The reaction mixture contain immobilized chitosanase: chitosan ratio 0.95 U/mg in acetate buffer, pH 5.6. It was incubated for 1.5 h at 55 °C. The resulted chitoooligosaccharides were separated by cooling centrifugation. Four chitoooligosaccharides groups (chito 1,2,3,4) with different molecular weights > 100, 100 to 10, 10 to 1 and < 1 KDa, respectively, were prepared by ultrafiltration. All fractions were lyophilized and stored at -4 °C.

2.4. Determination of the prepared chitoooligosaccharides

The prepared chitoooligosaccharides concentrations were estimated by dinitrosalicylic acid (DNS) method (Miller, 1959) using D-glucosamine as standard. Equal volume of chitoooligosaccharides and DNS reagent (3 ml) was heated for 15 min in boiling water bath and then 1.0 ml of 40% Rochelle salt solution was added. The intensity of brownish-red colour was measured using spectrophotometer at 575 nm.

2.5. Preparation of microorganisms

The pure cultures of organisms were sub-cultured in nutrient agar media. It consists of 20.0 g agar, 3.0 g beef extract, 5.0 g peptone and 3.0 g sodium chloride in 1.0 L distilled water at pH 7.0. Tested microorganisms were inoculated separately into the nutrient media and kept at 37 °C for 24 h then, they were kept at 4 °C until use.

2.6. Antimicrobial activity (MIC)

The minimum inhibitory concentration (MIC) values of chito towards the tested microorganisms were assessed by broth dilution method. Minimum inhibitory concentrations (MICs) were determined as the lowest concentrations of chito at which microorganisms cannot grow in Müller-Hinton (M-H) broth based on the method of Ruparella et al. (2008). Mueller-Hinton broth was prepared from a commercially available dehydrated base according to the manufacture's instruction. All tested chito 1, 2, 3 and 4 were serially diluted four fold named 1, 2, 3,8 tube number with Muller-Hinton broth to give final concentrations ranging from 6.66 to 0.00041 mg/ml (Table 1). Each tested

microorganism were inoculated in each tube. The tubes were incubated at 37 °C with shaking for 24 h. Bacterial growth (turbidity) was examined by measuring optical density at 640 nm. Antimicrobial activity experiments were repeated three times.

2.7. Specific growth rate

Specific growth rates (R) of bacteria or yeast strains were measured in the presence of chito (1, 2, 3 and 4) and other standard antibiotics (flomox and Kluacid) according to the method of Simunek et al. (2010). It was calculated by formula $R = (\ln x - \ln x_0) / (t - t_0)$, where x (x₀) is the turbidity (starting turbidity) and t (t₀) time period (zero time).

2.8. Statistical analysis

The results were expressed as a mean ± SD, n = 3 (standard deviation) for each analysis.

3. Results and discussion

Chitoooligosaccharides (chito) had been found to be an economical antimicrobial agents (Gerasimenko et al., 2004; Wang et al., 2007; Kulikow et al., 2014). They had advantage of no producing major side effects as is found in all case of usual antibiotics. In the present study, we have used a series of well-characterized (chito) groups, 1, 2, 3 and 4 with molecular weight >100, 10 to 100, 1 to 10 and < 1 KDa, respectively. They were prepared by enzymatic hydrolysis of chitosan using immobilized pepper chitosanase. Their characterization and preliminary (screening) antimicrobial activity were studied using growth inhibition zone method (El-Sayed et al., 2017). They had positive antimicrobial activities. This study was focus on determination of the MIC of the chito fractions upon three pathogenic microorganisms [Gram positive bacteria (*B. cereus*), Gram negative fungi (*P. aeruginosa*) and Gram positive yeast (*C. albicans*)] and compared them with that of standard broad spectrum antibiotic flomox and kluacid (Table 2). The minimum inhibition concentration (MIC) was determined by broth dilution method.

Results in figure (1) showed that in presence of chito 1, 2 and 3, the specific growth rate of *B. cereus* was completely inhibited at MIC of 0.026, 0.42 and 0.11 mg/ml, respectively, while kluacid and flomox showed MIC of 0.11 and 0.00041 mg/ml, respectively (Fig. 2). It can easily be observed that MIC did not depended on the molecular weight of chitoooligosaccharides. Chito (3) with 1–10 KDa molecular weight showed equal antimicrobial activity against *B. cereus* to that of broad spectrum antibiotic kluacid, while chito (1) with high molecular weight >100 KDa showed higher antimicrobial activity (low MIC 0.026 mg/ml) than that of other chito groups and antibiotic kluacid. Flomox showed lower antimicrobial activity (high MIC 1.67 mg/ml) than that of the three chito group. *B. cereus* is a Gram-positive bacteria that is widely distributed in nature. It is one of the most frequent food-poisoning microorganisms causing both intoxications and infections (Fernandes et al., 2009). They grow in food that has been improperly stored. They are considered a relatively common cause of gastroenteritis worldwide, causing vomiting and diarrhoeal. Antimicrobial chito has been found to

Table 1

Concentration of tested samples (chito and antibiotics) in each tubes number.

Tube number	Concentration of sample (mg/ml)
1	6.66000
2	1.67000
3	0.42000
4	0.11000
5	0.02600
6	0.0065
7	0.0016
8	0.00041

Table 2

Effect of different chito groups on antimicrobial activity (MIC) against the tested microorganisms.

Tested microorganisms	Samples	Molecular weight (KDa)	Tube number	MIC (mg/ml)
<i>Bacillus cereus</i>	Chito (1)	>100	5	0.026
	Chito (2)	10–100	3	0.42
	Chito (3)	1–10	4	0.11
	Flomox	<1	2	1.67
	Kluacid	<1	4	0.11
<i>Pseudomonas aeruginosa</i>	Chito (2)	10–100	2	1.67
<i>Candida albicans</i>	Chito (4)	<1	4	0.11
	Flomox	<1	8	0.00041
	Kluacid	<1	3	0.42

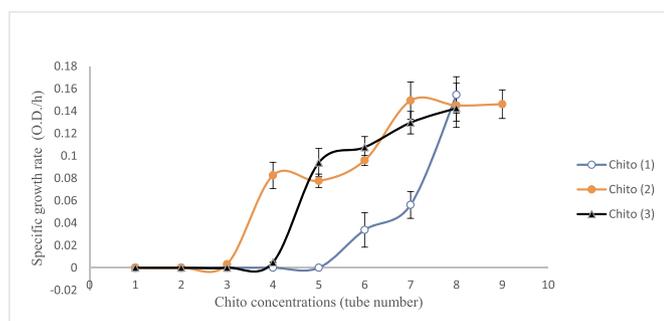


Fig. 1. The relation between the specific growth rate of *Bacillus cereus* and the different concentrations of chito (1), (2) and (3). Specific growth rate per hour = $(\ln x - \ln x_0)/(t - t_0)$, where x (x_0) is the turbidity (starting turbidity) and t (t_0) time period (zero time).

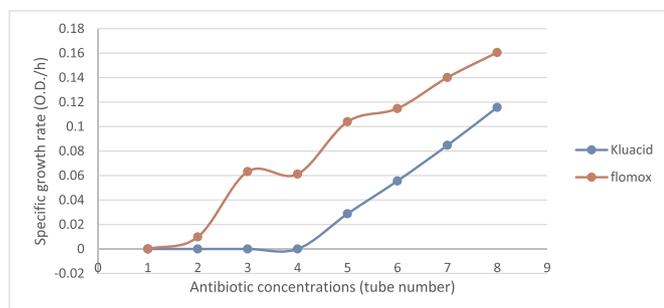


Fig. 2. The relation between the specific growth rate of *Bacillus cereus* and the different concentrations of broad spectrum antibiotic kluacid and flomox. Specific growth rate per hour = $(\ln x - \ln x_0)/(t - t_0)$, where x (x_0) is the turbidity (starting turbidity) and t (t_0) time period (zero time).

be an economical way to prevent (or treat) food poisoning caused by *B. cereus* (Jeon et al. (2001)). The growth of most bacteria tested by Jeon et al. (2001), was inhibited by chito oligosaccharide treatments, in particular by 10 KDa molecular weight. Chito (<3 KDa), on the other hand, provoked more visible damages in the *B. cereus* vegetative form—most probably due to the penetration of the cells by the chito oligosaccharides (Fernandes et al., 2009).

Chito (2) with 10–100 KDa should MIC 1.67 mg/ml against *P. aeruginosa* (Fig. 3). Chito (4) with molecular weight less than 1.0 KDa showed strong inhibitory effect (MIC of 0.11 mg/ml) against *C. albicans* than that of broad spectrum antibiotic kluacid (0.42 mg/ml), while in case of broad spectrum antibiotic flomox, the growth of *C. albicans* was complete inhibited (Figs. 4 and 5). *Pseudomonas aeruginosa* and *Candida albicans* were isolated from skin microflora. They can be found in the oral cavity, throat, gastrointestinal tract, vagina, nails, and skin (Sugar

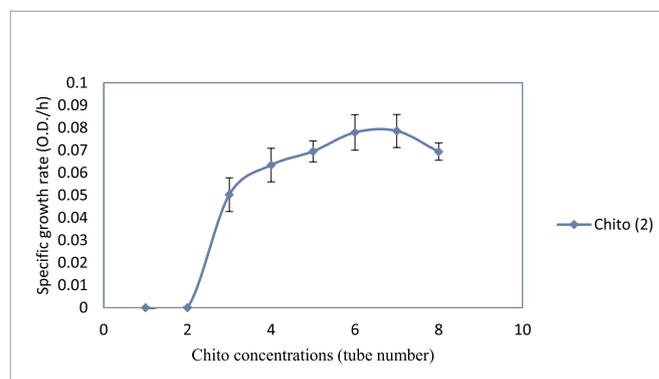


Fig. 3. The relation between the specific growth rate of *Pseudomonas aeruginosa* and the different concentrations of chito (2). Specific growth rate per hour = $(\ln x - \ln x_0)/(t - t_0)$, where x (x_0) is the turbidity (starting turbidity) and t (t_0) time period (zero time).

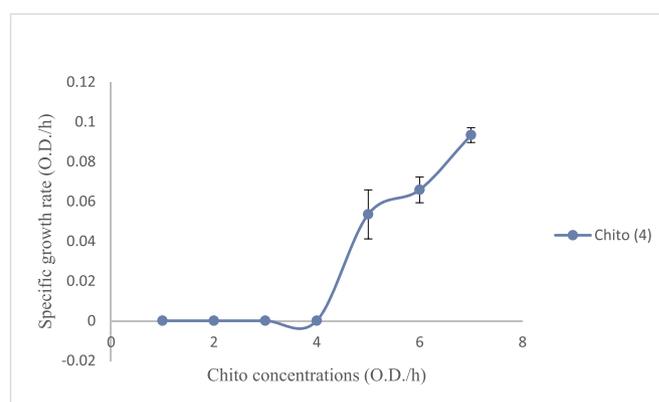


Fig. 4. The relation between the specific growth rate of *Candida albicans* and the different concentrations of chito (4). Specific growth rate per hour = $(\ln x - \ln x_0)/(t - t_0)$, where x (x_0) is the turbidity (starting turbidity) and t (t_0) time period (zero time).

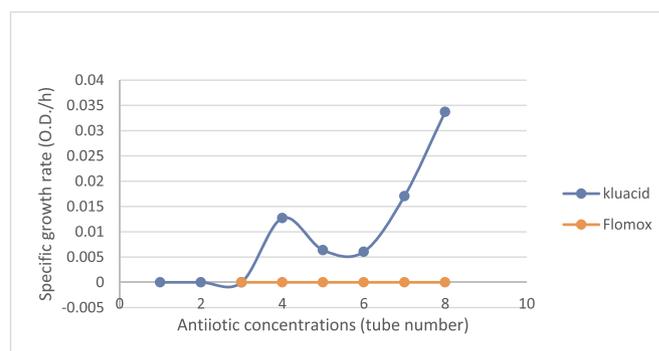


Fig. 5. The relation between the specific growth rate of *Candida albicans* and the different concentrations of broad spectrum antibiotic kluacid and flomox. Specific growth rate per hour = $(\ln x - \ln x_0)/(t - t_0)$, where x (x_0) is the turbidity (starting turbidity) and t (t_0) time period (zero time).

and Lyman, 1997). *Candida albicans*, is consider one of the most wide-spread ones. Kulikow et al. (2014) reported that chito having MW in the range 5–10 KDa possessed maximal activity in comparison with lower and higher chito. In another study, Ueno et al. (1997) have reported that chito oligosaccharides with an average molecular weight less than 2.2 kDa, was not capable of suppressing the microbial growth. Aam et al. (2010) and Seyfarth et al. (2008) found that chito with 4.6 kDa had a

considerable potential as antifungal activity. A strong antifungal effect of chito was detectable upon *C. Albicans* (Fernandes et al., 2010).

In comparing the antimicrobial activity of chito 2 against Gram positive *B. cereus* with MIC of 0.42 mg/ml (Fig. 1) and Gram negative *P. aeruginosa* with MIC of 1.67 mg/ml (Fig. 3), it can easily be observed that MIC depend largely on the type of target microorganism. Similarly, Fernandes et al. (2010) reported that the antimicrobial effect is dependent on the type of target microorganism. However, (Xia and Wu (1996); No et al. (2002); Zheng et al. (2003) reported that chito exhibits higher antibacterial activity against Gram-negative than Gram-positive bacteria.

Chito could be applied in pharmaceutical, cosmetic and food products. They considered as safe, non-toxic easily soluble in aqueous media.

4. Conclusion

Our results showed that, chito had high antimicrobial effects based on the MIC determination. They could be considered as a possible alternative/additive antimicrobial agents in pharmaceutical compositions. They may be employed as an ingredient in bactericidal industries as a potent antimicrobial agents.

Declaration of competing interest

There are no conflicts of interest.

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