J Parasit Dis (Jan-Mar 2016) 40(1):75–80
DOI 10.1007/s12639-014-0449-z

ORIGINAL ARTICLE

The anti-giardial effectiveness of fungal and commercial chitosan against *Giardia intestinalis* cysts in vitro

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Received: 2 August 2013 / Accepted: 19 February 2014 / Published online: 12 March 2014

Abstract Chitosan with poly-*N*-acetylglucosamine sequences is a deacetylated derivative of chitin that can be found in the exoskeletons of crabs, shrimp and lobsters, the cuticles of insects and the cell walls of fungi. The aim of the present study was to compare the effects of fungal chitosan (FC) prepared from the cell walls of *Penicillium viridicatum* and *Penicillium aurantiogriseum* with commercially available chitosan (CC) against *Giardia intestinalis* cysts in vitro. The giardia cysts were isolated using a sucrose method. Four concentrations (50, 100, 200 and 400 µg/ml) of each type of prepared chitosan were applied for 10, 30, 60 and 180 min. The viability of the cysts was checked via 0.1 % eosin staining. Our results indicate that *P. viridicatum* (with a 47.5 % DD) and *P. aurantiogriseum* (with a 47.3 % DD) at different concentrations after 180 min precipitated, respectively, 56, 69, 81 and 100 %, and 63, 75, 86 and 100 % mortality rates. CC (with a 54 % DD) showed 79, 84, 93 and 100 % mortality rates. In conclusion, both FC and CC at 400 µg/ml concentrations after 180 min of exposure showed the most potent effect against *G. intestinalis* cysts. Accordingly, chitosan could be suggested as a new natural nanoform agent for future research in the safe and effective treatment of *Giardia* infections.

Keywords *Giardia intestinalis* · Giardiasis · Chitosan · Fungi · *Penicillium* spp.

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Introduction

Parasitic infections are the primary causes of gastrointestinal syndromes in developing countries. Among the parasitic gastrointestinal infections, *Giardia intestinalis* is the most common (Faubert 2000). *Giardia intestinalis* is a flagellate protozoan that has a simple life cycle that is composed of two stages: the trophozoite stage and the cyst stage (infective form). The cyst is essential for the survival of the parasite outside of the host; new infections occur through the transmission of the cyst from host to host through fecal contamination (Svard et al. 2003; Adam 2001; Craun 1996; Guy et al. 2004). The clinical manifestations of this parasite are divided into asymptomatic giardiasis (carriers) or symptomatic giardiasis syndrome (diarrhea, nausea, abdominal pain, malabsorbtion and chronic giardiasis (a smaller number of cases, also resistant to treatment)) (Hill 2005; Ford 2005). Current chemical drugs, such as metronidazole, tinidazole and furazolidon, are frequently prescribed by clinicians for the treatment of giardiasis. These drugs have several side effects and a

limited utility due to drug resistance; thus, it is necessary to pursue natural and safe drugs that have strong therapeutic effects, low costs and minimal side effects (Wright et al. 2003). Chitosan is a partially or fully deacetylated chitin (Fig. 1) that exists in crustacean shells, insect cuticles and fungi cell walls. Chitosan has extensive applications in agriculture, food, biotechnology, cosmeticology and pharmaceutical industries. It has a very safe toxicity profile and is a promising excipient for the pharmaceutical industry (Qi et al. 2004).

Due to its potential widespread availability, chitosan from fungal cell walls could significantly decrease the cost of medication. To the best of our knowledge, this is the first study on fungal chitosan (FC) in use against *Giardia* cysts. In this investigation, we determined the effect of chitosan isolated from the cell wall of *Penicillium viridicatum* and *Penicillium aurantiogriseum* on the viability of *Giardia* cysts.

Materials and methods

Collection of *G. intestinalis* cysts

G. lamblia cysts were isolated from the stool samples of giardiasis patients. All samples were administered directly after arrival. A highly purified cyst suspension was achieved by combining the sucrose flotation method with a simplified sucrose gradient method (Sheffield and Bjorvatn 1977). The stools were broken up in normal saline and filtered through a 300 urn filter. A total of 3 ml of the stool suspension was layered on top of 3 ml of 0.85 M sucrose and centrifuged at 600×*g* for 10 min at 4 °C. The cysts were aspirated with a Pasteur pipette at the sucrose-water interface and washed 3 times with normal saline. The washed cysts were carefully added to the top of a discontinuous density gradient consisting of two 3-ml lavers of 0.85 M and 0.4 M sucrose. After centrifugation, the cysts concentrated at the 0.85–0.4 M sucrose interface were collected and washed again. The purified cysts were suspended in normal saline and stored at 4 °C for a maximum of 3 days prior to use.

Detection of the FC and CC antigiardial activities

The chitosan extracted from two *Penicillium* species (*P. viridicatum* and *P. aurantiogriseum*) in our previous study (Ebrahimzadeh et al. 2013) was prepared in four concentrations (50, 100, 200 and 400 µg/ml). For each concentration, incubation times of 10, 30, 60 and 180 min, respectively, were tested. A total of 2 ml of each solution was placed in test tubes, to which 10,000 washed cysts were added. The contents of the tubes were gently mixed. The tubes were then incubated at 37 °C for 10, 30, 60 and 180 min. At the end of each incubation period, the upper phase was carefully removed. A total of 2 ml of 0.1 % eosin stain was then added to the remaining settled cysts, which were then gently mixed. The cysts were then smeared on a glass slide, covered with a cover glass and examined under a light microscope. The percentages of the dead cysts were determined by counting 1,000 cysts per slide. Untreated cysts were used as a control group in each experiment. The tests were performed in triplicate.

Viability test

Eosin 0.1 % stain was used to determine the viability of the cysts. Fifteen minutes after exposure to the stain, the cysts with no absorbed dye were recorded as being potentially viable; otherwise, the cysts were recorded as dead.

Statistical analysis

The results were performed using SPSS software version 16 (SPSS Inc., Chicago, IL). Differences between the untreated and treated samples were tested for significance using a *T* test (a *P* value <0.05 was considered significant).

Results

The antiprotozoal activity of the isolated chitosan from *P. aurantiogriseum*, *P. viridicatum* and commercially available chitosan are shown in Tables 1, 2 and 3. Our data indicate a significant difference between the untreated

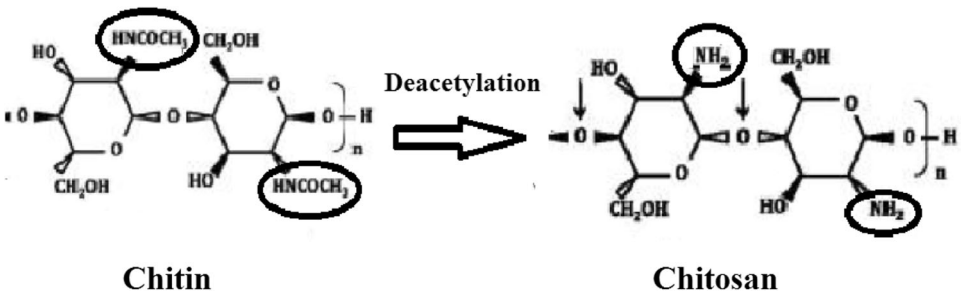

Fig. 1 Structures of chitin and chitosan

group and the group treated with chitosan for certain durations (30, 60 and 180 min) ($P < 0.05$). Time as an effective parameter influenced the mortality rate of *G. intestinalis* cysts; increasing the duration of contact also increased the mortality rate ($P < 0.05$). The difference between the groups treated with FC and the group treated with commercial chitosan was not statistically significant ($P > 0.05$). There were also no significant differences between the treated and untreated groups at 10 min of exposure ($P > 0.05$). A high mortality rate was observed at all concentrations after 180 min of contact. The mortality rate of the cysts treated with commercial chitosan, *P. viridicatum* or *P. aurantiogriseum* after 180 min of exposure at a concentration of 50 µ/ml was 56, 65 and 79 %, at 100 µ/ml was 69, 75 and 84 %, at 200 µ/ml was 81, 86 and 93 % and at 400 µ/ml was 100, 100 and 100 %, respectively. The mortality rate of the control group at 10, 30, 60 and 180 min was 6, 7, 7 and 9 %, respectively. As shown in Table 1, the commercial chitosan with a 54 % deacetylation rate shows the highest antigiardial action at all concentrations with a mortality rate of 79–100 % after 180 min. As shown in Fig. 2, when the rate of the deacetylation of CC was 54 %, the antigiardial activity of chitosan was at its highest point, while the *P. viridicatum* and *P. aurantiogriseum* (with 47.5 and 47.3 % DD) chitosan showed a weakened effect compared with the commercial chitosan. However, this difference was not significant.

Discussion

The present study demonstrated the chitosan prepared from two fungal species and its antigiardial activity against *G. intestinalis* cysts in vitro. Giardiasis is an important health issue that has a worldwide distribution and is particularly present in Iran. At present, chemotherapy remains a common treatment for giardiasis. However, resistance to chemotherapy is the primary problem in giardiasis treatment. Therefore, there is a pressing need for a low-cost drug with rapid and efficacious antigiardial action that has no toxicity or side effects (Kumar et al. 2007). FC appears to be an effective and safe alternative for the treatment of resistant infections. Several experiments demonstrated the use of herbal extracts as antigiardial agents. Rahimi-Esboei et al. (2012) reported that 100 mg/ml of hydroalcoholic extract of *Artemisia annua* had the highest cytotoxicity effect against *G. intestinalis* cysts after 24 h in vitro. In another study, they established that a concentration of 100 mg/ml of *Sambucus ebulus* had the highest antigiardial activity after 60 min (78 ± 4 %) (Rahimi-Esboei et al. 2013a, b). Said et al. (2012) investigated the antiparasitic potential of chitosan isolated from the larvae of *Musca domestica* against *G. intestinalis* cysts in vivo. They also reported that the antiparasitic activity of curcumin (a natural polyphenolic compound extracted from turmeric root) is not acceptable. Finally, they suggested that a combination therapy using the nanoforms of chitosan, silver and curcumin led to complete (100 %) trophozoite eradication. A number of synthetic and natural components have been noted for their intrinsic antigiardial action. However, many complications may occur due to their toxicities and carcinogenicities (Clark 1996). Chitosan (given its non-toxic, non-carcinogenic, non-allergen profiles) could be used in several applications such as anticoagulation, wound healing, microbiology, drug delivery, oral vaccine carrier, film coating, mouthwash, food and nutrition, agriculture, cosmetics, etc. Many in vivo and in vitro studies have confirmed the non-toxicity of chitosan to normal human cell lines, and the FDA has approved chitosan for clinical applications (Ariani et al. 2009). Chitosan is a well-known biopolymer that is widely used as an antimicrobial agent in pharmaceutical industries. Previous literature has focused on the antibacterial activity of chitosan from shrimp, crabs and fungi, and little information is available on the antiparasitic potential of FC. Tayel et al. (2011) investigated the antibacterial and antifungal activity of the derivatives of isolated chitosan from the biomass of *Aspergillus niger*. They considered chitosan-treated fabrics to be appropriate for medical use. Martinez et al. (2010) demonstrated the potential of isolated chitosan from crustacean exoskeletons for killing the pathogenic fungus *Cryptococcus neoformans*, which causes infections by forming a biofilm on indwelling medical devices. They also established that the chitosan concentration needed for the treatment of fungal

Table 1 The mortality rate of *G. intestinalis* cysts in the presence of different concentrations of commercially available chitosan and given different exposure times

Exposure time/concentration	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	*P* value	Control group
10 min	7	9	13	19	0.155	7
30 min	29	31	47	53	0.011	9
60 min	57	63	69	76	0.001	9
180 min	79	84	93	100	0.0001	8
P value	0.081	0.056	0.027	0.013	–	–

Table 2 The mortality rate of *G. intestinalis* cysts in the presence of different concentrations of *P. Viridicatum* chitosan and given different exposure times

Exposure time/concentration	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml	*P* value	Control group
10 min	6	9	15	19	0.175	7
30 min	23	29	39	49	0.016	9
60 min	38	49	61	69	0.005	9
180 min	56	69	81	100	0.006	8
P value	0.108	0.053	0.02	0.012	–	–

Table 3 The mortality rate of *G. intestinalis* cysts in the presence of different concentrations of *P. aurantiogriseum* chitosan and given different exposure times

Exposure time/concentration	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml	*P* value	Control group
10 min	6	7	16	21	0.223	7
30 min	13	19	37	41	0.057	9
60 min	43	50	62	69	0.004	9
180 min	63	75	86	100	0.003	8
P value	0.155	0.101	0.018	0.012	–	–

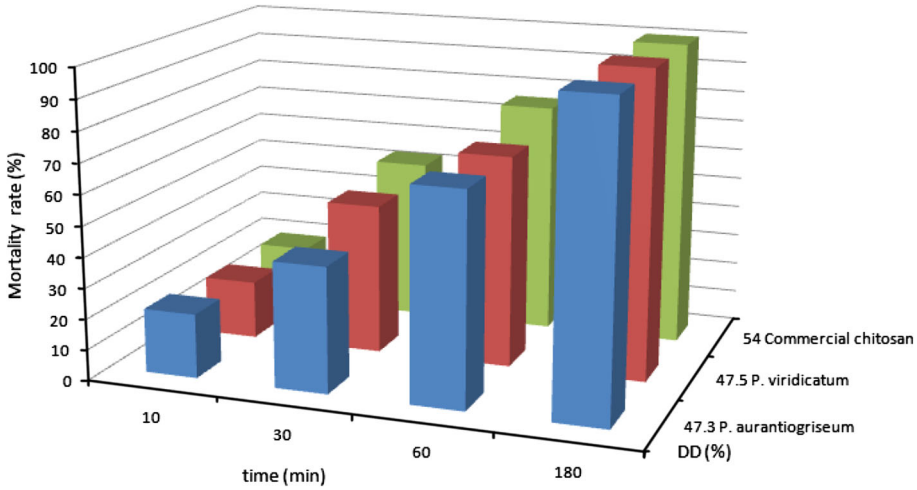

Fig. 2 Effect of the degree of deacetylation (DD) on the mortality rate of *Giardia intestinalis* cysts

biofilms shows no toxicity to human cells. In our previous investigations, we revealed that the FC prepared from *Penicillium waksmanii*, *Penicillium citrinum, P. viridicatum* and *P. aurantiogriseum* at concentrations of 400 μg/ml killed all *Echinococcus granulosus* cysts after 180 min (Fakhar et al. 2013; Rahimi-Esboei et al. 2013a, b). Many studies have demonstrated the efficacy of chitosan at different concentrations and exposure times for reducing the viability of different families of bacteria (*Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi-A*, *Vibrio parahaemolyticus*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, etc.) (No et al. 2002; Tin et al. 2009), fungi (*A. niger*, *Alternaria alternata*, *Alternaria solani*, *Phomopsis asparagi*, *Rhizopus stolonifer*, *Rhizopus oryzae*, *Phytophthora capsici*, *Phytophthora parasítica*, *Verticillium dahlia*, *Colletotrichum orbiculare*, *Exserohilum turcicum*, *Pyricularia oryzae*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium graminearum*, *Fusarium sulphureum*, *Fusarium solani*, *Candida albicans*, *Neurospora crassa*, *Penicillium expansum*, *Penicillum digitatum*, etc.) (Reddy et al. 1998; Guerra-S′anchez et al. 2009; Zhong et al. 2007; Ziani et al. 2009; Falcón et al. 2008; Kong et al. 2010; Yong-cai et al. 2009; Lafontaine and Benhamou 1996; Lauzardo 2009; Rane and Hoover 1993; Ting et al. 2007; Xu et al. 2007; Palma-Guerrero et al. 2009; Pacheco et al. 2008) and parasites (*Trichomonas gallinae*, *E. granulosus*, *Cryptosporidium parvum*, *Philasterides dicentrarchi*, etc.) (Tavassoli et al. 2012; Fakhar et al. 2013; Brown and Emelko 2008; Parama et al. 2005).

Conclusion

This study demonstrates the capability of chitosan prepared from *P. viridicatum* and *P. aurantiogriseum* isolated from agricultural soils in Iran to inactivate *G. intestinalis* cysts. The maximal action was observed with a 400 µg/ml concentration after 180 min (100 % reduction in cyst viability). Commercially available chitosan was more effective than FC against *G. intestinalis* cysts. The viability of *G. intestinalis* cysts decreased by increasing the chitosan concentration and exposure time. The use of the degree of deacetylation (DD) as a main parameter could reduce the exposure time. Further experiments are required to evaluate other natural products, particularly in their nanoform, as alternative therapies against giardia infections.

Acknowledgments The authors would like to thanks the American Experts Journal scientists for their appropriate English language editing. This study was supported by a Grant from the Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran.

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