



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading

doi:10.1016/S2221-1691(13)60160-4

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In vitro treatments of *Echinococcus granulosus* with fungal chitosan, as a novel biomolecule

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PEER REVIEW

Peer reviewer

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Comments

The data obtained clearly demonstrated that the chitosan derived from fungi and commercial one was protoscolicidal *in vitro*. Chitosan as a cationic natural polysaccharide and biopolymer demonstrated to be safe for mammalian cells.

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ABSTRACT

Objective: To determine the antiparasitic activity of the isolated chitosan from *Penicillium viridicatum*, *Penicillium aurantiogriseum* and commercial chitosan against protoscolicidal of hydatid cysts were determined.

Methods: After isolating chitosan from fungal cell walls, four concentrations (50, 100, 200, 400 µg/mL) of each type of prepared chitosan and commercial chitosan were used for 10, 30, 60, and 180 min, respectively.

Results: Among different type of chitosan, commercial chitosan with the highest degree of deacetylation showed high scollicidal activity *in vitro*. Fungal chitosan could be recommended, as good as commercial chitosan, for hydatid cysts control.

Conclusions: It seems to be a good alternative to synthetic and chemical scollicidal.

KEYWORDS

Chitosan, Protocoleces, *Echinococcus granulosus*, *Penicillium*

1. Introduction

Echinococcosis is a zoonotic infection caused by adult or larval (metacestode) stages of cestodes belonging to the genus *Echinococcus* and the family Taeniidae[1]. *Echinococcus granulosus* (*E. granulosus*) produces unilocular cysts characterized by a thick, multilayered cyst wall with a thin, internal germinal membrane; protoscolices and daughter cysts, containing more protoscolices, may form internally; cysts are contained by a connective tissue capsule[2]. Hydatid cysts in humans can cause liver dysfunction or respiratory disease, and anaphylaxis occurs if the cysts rupture. For every hydatid cyst ingested by the definitive host, many worms can develop[3,4]. Surgery is still the main treatment for hydatid disease[5]. But several groups

of drugs including cytostatics, antibiotics, sulphonamides, antiprotozoal compounds and several antihelminthic drugs have been tested for their efficacy against the metacestode stage of *Echinococcus*[6]. Chitosan is a partially or fully deacetylated chitin, which exists in crustacean shells, cuticles of insects, and fungi cell wall[7].

Chitosan has been used in broad spectrum of agriculture, food, biotechnology, cosmeticology and pharmaceutical industries[8]. Chitosan is of unique biological characteristics such as biodegradability, biocompatibility and non-toxicity in mammalian cells[9]. Antifungal and antibacterial activity of fungal chitosan has also been observed against several fungi and bacteria, but a few researches have been carried out on fungal chitosan antiparasitic activity[10–13]. The aim of our investigation was to evaluate scollicidal

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Foundation Project: This work was supported by a grant from Student Research Committee of Mazandaran University of Medical Sciences, Sari, Iran (Grant No. 91/150).

Article history:

Received 12 Jul 2013

Received in revised form 20 Jul, 2nd revised form 29 Jul, 3rd revised form 7 Aug 2013

Accepted 5 Sep 2013

Available online 28 Oct 2013

activities of various concentrations of different types of fungal chitosan isolated from *Penicillium viridicatum* (*P. viridicatum*), *Penicillium aurantiogriseum* (*P. aurantiogriseum*) in comparison to commercial one against protoscolices of *E. granulosus in vitro* in different exposure time.

2. Materials and methods

2.1. Collection of protoscolices

Protoscolices were aspirated from the liver cysts of infected sheep slaughtered at Sari slaughterhouse, Mazandaran Province, in Northern Iran. The hydatid fluid was transferred into test tube under sterilized condition and set for 30 min. The sedimental protoscolices were washed with normal saline for three times. The viability of the protoscolices was assessed by eosin 0.1% under a light microscope. The protoscolices those did not absorb dye were considered potentially viable, otherwise recorded as dead.

2.2. Extraction of chitosan

Low molecular weight commercially chitosan (CC) was purchased from Fluka. Two *Penicillium* species, *P. aurantiogriseum* and *P. viridicatum* from the department of mycology at Mazandarane Agriculture University, were used as a source of fungal chitosan. The fungal chitin (FC) was deacetylated by a modified method of Rane and Hoover^[14]. After cultivation of fungal mycelia, the dry biomass were suspended with NaOH solution (1 mol) and autoclaved at 121 °C for 20 min. The alkaline undissolved fractions were collected by centrifugation at 12000 r/min for 20 min, washed with distilled water and recentrifuged to obtain pH=7. The precipitation was further extracted using 2% acetic acid at 95 °C for 8 h. The insoluble particles suspension was centrifuged, then the supernatant solution was neutralized with NaOH (2 mol/L), the solution centrifuged and the precipitated chitosan was washed with distilled water.

Degree of deacetylation (DD) of FC and CC was measured by FT-IR spectroscopy (Perkin Elmer Pergamon 781, from 4000 to 450) and the calculation proposed as $DD=100-[(A_{CO}/A_{OH})\times 100/1.33]$ ^[15]. To prepare chitosan solutions, each prepared chitosan sample from *Penicillium* species and CC was dissolved in 1% (w/v) acetic acid solution and then the solution was neutralized by 1 mol NaOH (medium).

2.3. Scolicidal assay

In our study, four concentrations (50, 100, 200, 400 µg/mL) of the chitosan were prepared. Then 10000 washed protoscolices were added into 2 mL of each chitosan solutions and softly mixed. The mixed solution was incubated at 37 °C for 10, 30, 60, 180 min. Two milliliters of 0.1% eosin was then added to

the sediment protoscolices. The pellet of protoscolices was examined under a light microscope. The percentages of dead cysts were determined by counting 500 cysts. No treated cysts were considered as a control group in each experiment. The experiments were performed in three times.

2.4. Statistical analysis

Triplicate trials were done for each experiments and statistical analysis for all the results was carried out using SPSS software. One sample *t*-test was performed to define significant variation between control and chitosan solutions at $P<0.05$.

3. Results

The scolicidal activity of FC and CC at different concentrations (50, 100, 200, 400 µg/mL) after 10, 30, 60 and 180 min of exposure time in comparison with control group are showed in Tables 1, 2 and 3.

Table 1

Scolicidal effect of different concentrations of prepared chitosan from *P. viridicatum* on the mortality rate of protoscolices of hydatid cyst at various exposure times.

Exposure time (min)	Concentration (µg/mL)				Control group
	50	100	200	400	
10	7	9	19	27	7
30	26	35	47	58	7
60	47	56	66	71	8
180	63	76	86	100	9

Table 2

Scolicidal effect of different concentrations of prepared chitosan from *P. aurantiogriseum* on the mortality rate of protoscolices of hydatid cyst at various exposure times.

Exposure time (min)	Concentration (µg/mL)				Control group
	50	100	200	400	
10	5	7	19	28	7
30	15	25	41	46	7
60	49	53	62	70	8
180	79	76	83	100	9

Table 3

Scolicidal effect of different concentrations of commercially chitosan on the mortality rate of protoscolices of hydatid cyst at various exposure times.

Exposure time (min)	Concentration (µg/mL)				Control group
	50	100	200	400	
10	8	11	16	29	7
30	33	39	51	59	8
60	60	63	69	78	9
180	89	93	97	100	9

The maximum DD was obtained by CC (54%) and the

minimum of this was 47.4% for *P. viridicatum* and *P. aurantiogriseum* respectively. No treated hydatid cyst is shown in Figure 1. Also, the dead protoscolex after treatment with chitosan is indicated in Figure 2. The efficacies of the deacetylation degree in toxicity of chitosan on hydatid cyst are shown in Figure 3. The hydatid cyst was susceptible after treatment in all of the concentrations. Protoscolex was more sensitive to CC than FC since CC at the lowest concentration indicated higher mortality rate. The result of our study demonstrated that the scolicedal effect at any concentrations of CC and FC shows significant difference compared with control groups ($P=0.033, 0.018, 0.008, 0.006$). Also, the scolicedal activity of CC and FC after 30, 60 and 180 min was significant compared with the control groups ($P=0.016, 0.001, 0.001$), but it was not significant after 10 min. It needs 100 $\mu\text{g}/\text{mL}$ FC and CC after 60 min to reduce approximately 50% of the viable protoscolices. CC with higher DD (54%) showed higher mortality rate compared to FC ranging from 89% to 100% after 180 min of exposure time at all chitosan used dosages. The maximum mortality rate (100%) was achieved at 400 $\mu\text{g}/\text{mL}$ concentration after 180 min exposure time.

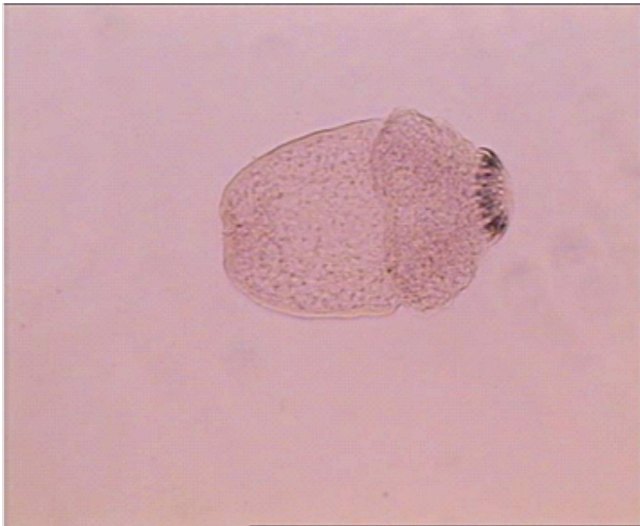


Figure 1. Viable protoscolices with no absorbed dye (control group).



Figure 2. Dead protoscolices with absorbed dye (after treatment with chitosan).

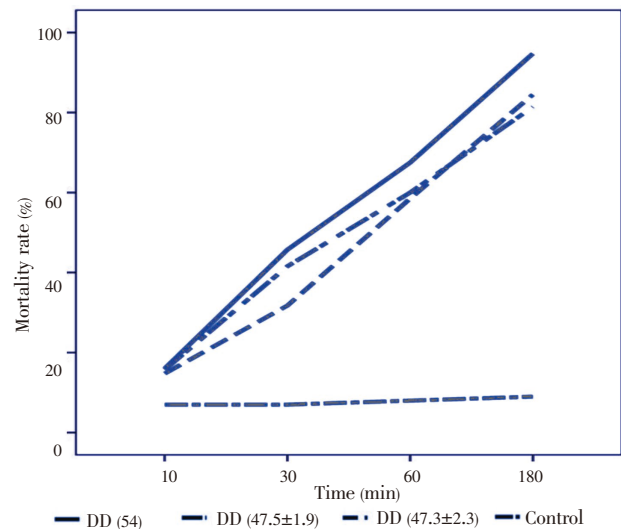


Figure 3. Effect of DD on the mortality rate of protoscolex.

4. Discussion

Chemotherapy, surgery, and puncture with aspiration are the three traditional treatments for hepatic hydatid cysts. If patients for whom surgery is not suitable or they refuse surgery, chemotherapy and puncture are recommended as alternative medication[16]. Inactivation of the scolex with a protoscolicedal agent before surgery is intensely recommended, because the spillage of the cyst contents is a major cause of return[17]. During hydatid cyst surgery protoscolicedal agents used to reduce the risk of spillage of viable protoscolices for surgical success[16]. The benzimidazole derivatives and mebendazole are common drugs presently used for management of hydatid cysts. And these drugs have been successful in many cases[18–21]. However, like other chemical agents, mebendazole has some side effects including liver toxicity or poor absorption[15]. Our study aimed to decrease the high risk of problem and recurrence during the surgery. Chitin and chitosan have a wide range of uses. These properties, together with the very safe toxicity profile, make chitosan an exciting and promising excipient for the pharmaceutical industry for present and future applications. Biodegradability and biocompatibility as some of their specific properties make them suitable for medical application. Chitin and chitosan are basic polymers mostly used in the preparation of medical biomaterials[9]. Presence of more than 0.025% chitosan inhibited the growth of *Escherichia coli*, *Fusarium*, *Alternaria* and *Helminth osporium*[22]. The combination of chitosan with drug make it possible to provide suitable extended release in gastrointestinal tract for intestinal *Cryptosporidium* infections[22]. Therefore, in order to reduce the bad effects of using chitosan, toxicity testing is needed. Many researches have been done to find the toxicity of chitosan[9,23,24]. Chitosan is widely regarded as a non-toxic, biologically compatible

polymer[25]. It is approved for dietary applications in Japan, Italy and Finland[26]. In this study, it was the first time to use fungal chitosan as an antiparasitic agent and our results at different concentrations (50, 100, 200, 400 µg/mL) after 10, 30, 60 and 180 min of exposure time showed high protoscolicidal effect of chitosan in comparison with control groups. The maximum mortality rate (100%) was achieved at 400 µg/mL concentration after 180 min exposure time. The parallel experiment showed that chitosan at the highest concentration (1250 µg/mL) and exposure time (360 min) could stop the viability of *Trichomonas gallinae* completely[27]. Commonly, the highest degraded chitosan serves a better inhibitory effect on *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces unisporus*, *Saccharomyces bayanus* and *Porphyromonas gingivalis*[28–30]. FC and CC seemed to be more effective against hydatid cyst contents rather than herbal component that discussed above, at the lowest concentration (0.02%) served a better killing effect on protoscolices (200 µg/mL after 180 min, 83%, 86% and 96%, respectively). The results have indicated that with the increase of chitosan concentration and exposure time, the viability of hydatid cysts decreased. DD as a main parameter could reduce the exposure time with the prolonged action. When DD of CC was 54%, the protoscolicidal activity of chitosan was at its highest point, while *P. viridicatum* and *P. aurantiogriseum* with 47.5% and 47.3%, DD indicated weaker protoscolicidal effect than commercial one. However, there is no significant difference between CC and FC. Uchida *et al.* found that chitosan with higher DD was more effective for inhibiting fungi and bacteria[31]. There is a correlation between antiparasitic effects and the content of protonated amino groups in chitosan structures. This may be attributed to the existence of NH_3^+ group which named DD. This parameter increased positive charge of chitosan molecule which interacts with the negative charge of parasite plasma membrane, upsetting cell membrane and promoting the leakage of intracellular constituent. To administer of chitosan in combination to other agents, it might reduce the drug resistant, complications, and obtained much shorter periods of treatments. In conclusion, the data obtained clearly demonstrated that the chitosan derived from fungi and commercial one was protoscolicidal *in vitro*. Chitosan as a cationic natural polysaccharide and biopolymer was demonstrated to be safe for mammalian cells. Hence, chitosan was recognized as a good candidate among natural protoscolicidal agents. In addition, the chitosan has been commonly available, easily prepared, and inexpensive. Therefore, it may be promising for hydatid disease. However, *in vivo* efficacy of chitosan on hydatids and the possible side effects need further investigation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by a grant from Student Research Committee of Mazandaran University of Medical Sciences, Sari, Iran (Grant No. 91/150).

Comments

Background

Echinococcosis is a zoonotic infection caused by adult or larval (metacestode) stages of cestodes belonging to the genus *Echinococcus* and the family Taeniidae. Chitosan is a partially or fully deacetylated chitin, which exists in crustacean shells, insects' cuticles and fungi cell wall. Chitosan has a wide range of uses. These properties, together with the very safe toxicity profile, make chitosan an exciting and promising excipient for the pharmaceutical industry for present and future applications.

Research frontiers

The aim of this investigation was to evaluate scolicidal activities of various concentrations of different types of FC isolated from *P. viridicatum*, *P. aurantiogriseum* in comparison to commercial one against protoscolices of *E. granulosus in vitro* in different exposure time.

Related reports

Hydatid cysts in humans can cause signs of liver dysfunction or respiratory disease, and anaphylaxis occurs if cyst ruptures. For every hydatid cyst ingested by the definitive host, many worms can develop. Chitosan has broad spectrum use as an antimicrobial agent.

Innovations and breakthroughs

Antifungal and antibacterial activity of FC has also been observed against several fungi and bacteria, but a few researches have been carried out on FC antiparasitic activity. Many of these scolicidal agent cause many original undesirable complications that limit their use. There is always a demand for finding natural materials with rapid and complete scolicidal effect with no local or systemic side effects as well as low cost. In this study authors demonstrated that FC has mentioned properties.

Applications

Chitin and chitosan have a wide range of uses. These properties, together with the very safe toxicity profile, make chitosan an exciting and promising excipient for the pharmaceutical industry for present and future applications. Biodegradability and biocompatibility as some of their specific properties make them suitable for medical application. Chitin and chitosan are basic polymers mostly used in the preparation of medical biomaterials.

Peer review

The data obtained clearly demonstrated that the chitosan

derived from fungi and commercial one was protoscolicidal *in vitro*. Chitosan as a cationic natural polysaccharide and biopolymer was demonstrated to be safe for mammalian cells. Hence, chitosan was recognized as a good candidate among natural protoscolicidal agents. In addition, the chitosan has been commonly available, easily prepared, and inexpensive. Therefore, it may be promising for hydatid disease.

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