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Immunomodulating Effect of Chitosan Extracted from *Aspergillus niger*

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Abstract: This study was conducted to extract chitosan from *Aspergillus niger* mycelia using hot alkaline followed by acid treatment and after 12 days of fermentation in sabouro dextrose broth containing 8% glucose, 1.246 g/L of chitosan was obtained. Five male albino Swiss mice were injected intraperitoneally with constant dose of chitosan (1 mg/mL) twice during two weeks and then blood picture, microscopic examination and double diffusion test were carried out. Chitosan was reported to increase the number of cells which were 530, 250 and 1237%, for Lymphocyte, Granulocytes and monocyte respectively when compared with control group. Also mice organs were crossly examined and they showed hypertrophy of liver and spleen, while microscopic examination reveled that spleen diffuse hyperplasia and infiltrate of mononuclear cells and megakaryocte. Moreover no antibody was detected in the serum of treated animals against chitosan.

Keywords: *Aspergillus niger*, chitosan, immunization, immunomodulator, leukocyte, polysaccharide

INTRODUCTION

Fungal mycelia wastes of the antibiotic or citric acid industry can become free and rich alternative sources of chitin-chitosan materials, beside the traditional industrial source-shellfish waste materials. Moreover, the fungal chitosans can have unique properties compared with those derived from Crustacea. There is a possibility of industrial production of chitosan from *Aspergillus niger* mycelia al wastes from citric acid production, The main part of the mycelial waste is discarded to the sewage treatment system or burnt. Since supplies of seafood waste are seasonable and variable, new research has been carried out on the use of alternative sources for chitosan (McGahren *et al*., 1984).

The studies were focused mainly on chitosan from fungi. The production and purifcation of chitosan, from the cell walls of fungi grown under controlled conditions, offer a greater potential for more consistent products (Niederhofer and Muller, 2004).

Chitosan is a cationic biocompatible polysaccharide built by repeated units of N-acetyl-D-glucosamine and D-glucosamine, derived from the partial deacetylation of chitin, a natural polysaccharide extracted from the crustacean shells. Chitosan is also found in some microorganisms in yeasts and fungi (Lllum, 1998). It has been administered to humans by several routes without toxic effects (Mills *et al*., 2003). This polysaccharide is able to activate leukocytes in vitro and in vivo (Porporatto *et al*., 2004; Peluso *et al*., 1994).

Over 20 years ago, chitosan, were found to be potent activators of macrophages and NK cells, Chitosan has comparatively better anti-microbial, anti-tumor and immune-stimulation biofunctionalitie (Nishimura *et al*., 1984, 1986).

The goal of this study was to determine the effects of extractable chitosan obtained from *Aspergillus niger* as immunostimulating agent for leukocytes when used as *In vivo* stimulants in mice.

MATERIALS AND METHODS

Extraction of chitosan from *Aspegillus niger* mycelia: Potato Dextrose Agar (PDA), NaOH and acetic acid were obtained from the Merk Company. The ethanol and acetone used in this study were of a commercial grade.

The fungus strain used in this study was A. Niger from the Biotechnology department, science collage-Baghdad University

PDA slants preparation: Potato Dextrose Agar (PDA) slants were prepared according to the manufacturer's instructions in order to cultivate the selected *A. niger*.

The spore of *A. niger* was inoculated on PDA slants and incubated at 30°C. After 3 days, the fungus growth on the PDA slants was stored at 4°C in a refrigerator. The sterile saline (9 g/L NaCl solutions) was poured into a tube and mixed well in order to bring the spore into a solution. Spores in the suspension were counted and the number was adjusted to 3×10^6 spores/mL/Sabouro Dextrose Broth (8% glucose) media were used for submerged fermentation as a medium. The content of glucose in the SDB was 8% 3×10^6 spores were inoculated into 250 mL sterilized flasks containing 50 mL of Sabouro Dextrose Broth (8%

glucose). The culture flasks were incubated at 30°C for 12 days at 150 rpm (Maghsoodi *et al*., 2009).

Chitosan extraction: The fungal mycelia were harvested and 50 mL of 1 N NaOH solution were added per gram (wet weight) of mycelia and homogenized. The content was sterilized at 121°C for 20 min (alkali treatment). The Alkali Insoluble Materials (AIM) were collected by centrifugation at 6000 rpm for 20 min and then washed several times with distilled water to neutralise them (pH 7). AIMs were dried in an oven at 40°C. They were then treated with acetic acid 2% (v/v), as a chitosan solvent, under a reux condition for 6 h at 95°C (1:30 w/v). The acid insoluble fraction was separated by centrifugation at 6000 rpm for 15-20 min and the supernatant containing the chitosan was isolated. The pH was adjusted with a 2 N NaOH solution in order to precipitate the fungal chitosan. The occulted chitosan was centrifuged at 6000 rpm, for 15 min. Isolated chitosan was washed four to five times with distilled water to neutralize it. At the same time, ethanol (96%) and acetone were employed to rinse the chitosan and then it was dried in a vacuum oven dryer at 60°C (Nwe *et al*., 2001; Chatterjee *et al*., 2005).

Immunomodulating effect of chitosan:

Immunization procedure: This procedure was done according to the method described by Kournikakis and Pilot-Ostroff (2002).

Five animals unless control was injected with 1 mg/ml of isolated chitosan intraperitoneally, after one week, each animal was injected with the same dose. The animals were scarified after one week of the last dose. Some organs (liver, Small intestine, Spleen) were taken and preserved with 10% formalin.

Blood smear was done for each animal to study the blood picture (Lymphocyte, Monocyte and Granulocyte), after stained by Giemsa stain. The percent of leukocytes calculation:

The percent of leukocytes determined in microscopically examination for animals were compared to control group using the following formula (Usˇan *et al*., 2006).

$$(L = \text{leukocytes}):\ L\% = \frac{\text{L ratio in experiment}}{\text{L ratio in control}} \times 100$$

Histological section: Histological section was done for spleen, liver and intestine.

Determination of antibody of chitosan in serum:

Double diffusion test (ouchterlony test): This method done according to Ouchterlony Double Diffusion (2012). Agarose (100 mg) was boiled in 10 mL of PBS buffer and cooled to 55°C then poured 5 mL of the gel solution on to a clean glass plate placed on a horizontal surface and allowed the gel to set, it takes approximately 20-30 min. The gel plate was placed on the template provided and punched wells in the gel with the help of a gel punch corresponding to the markings on the template. Used gentle suction to avoid forming rugged wells and took six vials and label as neat, 1:2, 1:4, 1:8, 1:16, 1:32, Serially diluted the test antiserum up to 1:32 dilution as follows:

(Took 20 µL of PBS buffer in each of the five vials (labeled from 1:2 through 1:32). Added 20 µL of test antiserum into the first vial and mixed well. The dilution of antiserum in this vial is 1:2. Transfer 20 µL of 1:2 diluted antiserums from the first vial in to the second vial. The dilution in this vial is 1:4 repeated the dilutions up to fifth vial then added 10 µL of the antigen to the center well and 10 µL each of neat (undiluted), 1:2, 1:4, 1:8, 1:16, 1:32 dilutions of antiserum into the surrounding wells. The plate was placed in a moist chamber and incubates at room temperature, overnight. After incubation, observe for opaque precipitin line between the antigen and antis era wells. The precipitin lines will be visible in 24-48 h.

RESULTS AND DISCUSSION

Extraction of chitosan from *Aspergillus niger*: Chitosan was extracted from the fungal mycelia using a hot alkaline flowed by acid treatment, after 12 days of fermentation, 1.246 g/L of chitosan was obtained. Sabouro Dextrose Broth was used for submerged fermentation as a medium containing 8% glucose to produce a chitosan under controlling conditions.

Different amounts of chitosan have been reported, about 1.8 g/L of chitosan was obtained with fungus *Abidia coerulea* using a Peptone-Glucose-Yeast extract medium (PGY) (Muzzarelli *et al*., 1994). While a 2.8 g/L yield using glucose, yeast and mineral media was reported (Davoust and Persson, 1992). The yield of chitosan produced in this work was lower than the production of chitosan which was obtained by Muzzarelli *et al*. (1994) and Davoust and Persson (1992). Due to the nature of the native fungus and the type of medium. Also In this study higher amount of chitosan was obtained than a yield of 0.47 g/L from *Gongronella butleri* (Tan *et al*., 1996). Other searchers experienced that the yields of isolated chitosan were 0.12 g/L of fermentation medium under liquid fermentation conditions (Crestini *et al*., 2002) and a 78.3 mg/L yield was reported by Hu *et al*. (2004) using PGY salt broth for *A. niger*.

Immunomodulating effect of chitosan: The immunmodulatory effect of chitosan was investigated five male albino swiss mice that were injected intraperitoneally, the results showed increased in the percentage of immune cells (lymphocytes, monocytes,

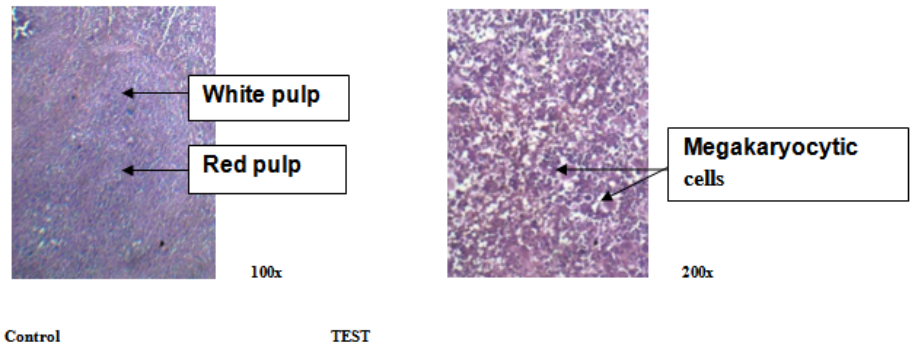

Fig. 1: Showed microscopic examination of spleen

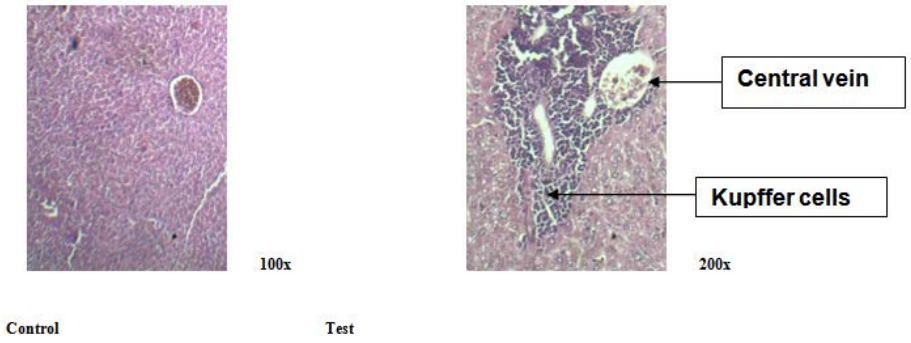

Fig. 2: Showed microscopic examination of liver

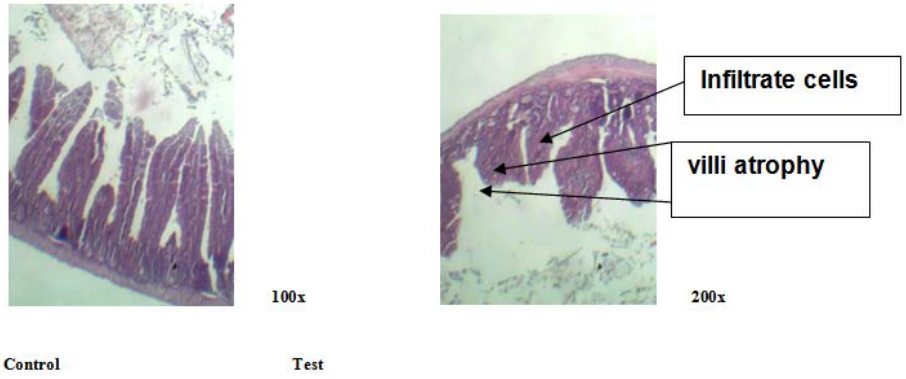

Fig. 3: Showed microscopic examination of intestine

granulocytes) when blood picture was examined which were higher than control after exposure to chitosan, the percentage of (lymphocyte was 530%, monocytes 1237% and granulocytes 250%), this was indicate that *A. niger* have active charbohydrate in the mycelia. On the other hand there were crossly enlargement in some organs like animals spleen, liver and several payers batches in the small intestine that mean there was immuno reaction on these organs due to the effect of the chitosan.

Otherwise, the results of microscopically examination were showed in spleen diffuse hyperplasia and infiltrate of mononuclear cells and megakaryocte cells compared with control which showed normal structure appearance with the of white and red pulp (Fig. 1).

While in liver tissue sectioning showing infiltration of mononuclear cells compared with control, normal structure of hepatocyte cells and central vein was seen (Fig. 2).

In intestine the sectioning tissue showing certain intestinal atrophy with infiltrate and inflammation cells inside the villi which compared with control which showing normal structure appearance of intestinal villi (Fig. 3).

Double diffusion test (ouchterlony test): This test was done to investigate the antibodies against chitosan in serum of treated mice but no results were obtained when this test was done. This may be due to that chitosan stimulate the leukocytes but no stimulation of immune cells to produce antibodies in serum.

CONCLUSION

From this study we can concluded that the extraction method was efficient in extraction of chitosan from the mycelia of *Aspergillus niger* and its an active compound in stimulating of cell mediated immunity but not the humeral immune responses.

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