

# The potentiality of using chitosan and its enzymatic depolymerized derivative chito-oligosaccharides as immunomodulators

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## ABSTRACT

Among the various methods used for the hydrolysis of chitosan, enzymatic hydrolysis was the most preferred method. Hence, in this research, the hydrolysis of chitosan to chito-oligosaccharides was performed using partially purified *Dothideomyces* sp. chitosanase with a specific activity 16.7 IU/mg protein in a reaction mixture contained enzyme/substrate ratio 0.2 IU/mg incubated for 1 hour at 45°C. The *in vivo* immunomodulatory effects of both chitosan and COS in low and high doses (10 and 20 mg/kg body weight) were studied using normal mice and lipopolysaccharide (LPS)-treated mice. In the case of their intraperitoneal injection in normal mice, they caused an increase in peritoneum cell infiltration, phagocytic activity of the peritoneal macrophage, and serum level of tumor necrotic factor- $\alpha$ , nitric oxide, lysozyme activity, and interleukin-6. Moreover, they significantly overcome LPS-induced severe activation of all the tested parameters. The results demonstrated that both had dual activities, immune-stimulatory activity and anti-inflammatory activity. Collectively, the present study suggests that chitosan and COS can be used as endotoxin scavenger and therapeutic agents in disorders where inflammation is one of the pathological features.

## INTRODUCTION

Chitosan is a polysaccharide consisting of  $\beta$ -(1 $\rightarrow$ 4)-2-acetamido-D-glucopyranose and  $\beta$ -(1 $\rightarrow$ 4)-2-amino-D-glucopyranose units, with the latter usually exceeding 80% (Zou *et al.*, 2016). It has been proved to be nontoxic, biodegradable, and biocompatible compound manufactured by the deacetylation of chitin that exists in the shells of the crustaceans, the insect exoskeletons, and in the cell walls of fungi and algae (Hamed *et al.*, 2016). It has three types of reactive functional groups, an amino/acetamido group, a primary and a secondary hydroxyl groups at the C-2, C-3, and C-6 positions, respectively, which are responsible for its various biological activities including antitumor activity, antimicrobial activity, antifungal activity, antioxidant, and immune-enhancing effects (Zou *et al.*, 2016). The biological activities of chitosan have been suggested to be

due to the activity of its hydrolysis products in the target tissues. So, hydrolysis of chitosan to chito-oligosaccharides (COS) is of a great interest due to the improved water solubility and lower viscosity that enhances its activity in many biological applications (Sanchez *et al.*, 2017).

COS are the partial hydrolysis products of chitosan with an average molecular weight up to 3900 Da (Hamed *et al.*, 2016). Several methods including depolymerization of chitosan under the action of reagents, enzymes, high-energy impact, and bioengineering are used for the preparation of COS. The enzymatic hydrolysis was the most specific, significant, safe, and environmental friendly method (Ismail, 2016; Pan *et al.*, 2016). Similar to the parent chitosan, COS are positively charged in aqueous environments that allow them to bind strongly to the negatively charged surfaces result in various biological activities (Liaqat and Eltem, 2018; Zou *et al.*, 2016).

Lipopolysaccharide (LPS) is the glycolipid constituent of the outer membrane of all pathogenic gram-negative bacteria that known as one of the most potent activators of the inflammatory responses. LPS activates the inflammatory signal transduction by the cell surface toll-like receptor-4 (TLR-4) that results in cellular activation and pro-inflammatory gene expression (Yang *et al.*, 2016).

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During the inflammation process, macrophages actively participate in the inflammatory responses by releasing the pro-inflammatory cytokines, tumor necrotic factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-6 (IL-6), as well as other inflammatory factors, such as the short-lived free radical nitric oxide (NO) and prostaglandins, that recruit additional immune cells to the sites of infection or tissue injury (Boscá *et al.*, 2005). Nitric oxide is endogenously synthesized by nitric oxide synthases (NOSs) through the conversion of L-arginine to NO and L-citrulline. Inducible NOS (iNOS) are the most abundant type that expressed and released in macrophages activated by LPS (Kim *et al.*, 2014).

Chitosan and COS are considered as immunomodulators since they stimulate the synthesis of some of the cytokines that cause either stimulation or inhibition of the immunity (Davydova *et al.*, 2016). These discrepancies lead to the attraction of the research focus. The present study concerned with the enzymatic hydrolysis of chitosan using chitosanase to produce COS then investigated the effect of chitosan and the produced COS on the immune status of normal mice as well as evaluating their anti-inflammatory effect on LPS-inflamed mice.

## MATERIALS AND METHODS

### Chemical

Chitosan [low (50–190 kDa) and medium (190–310 kDa) molecular weight], standard glucosamine, standard N-acetyl glucosamine, yeast extract, RPMI (Roswell Park Memorial Institute) 1640 media with L-Glutamine, fetal calf serum (FCS), *Micrococcus lysodeikticus* bacteria, agarose, sulphanilamide, and N-(1-naphthyl)- $\alpha$  naphthyl amine were obtained from Sigma-Aldrich, Saint Louis. Dinitrosalicylic acid was obtained from Panreac, Barcelona, Spain. Thin-layer chromatography (TLC) Silica gel 60 plate was obtained from Merck, Darmstadt, Germany. *Candida albicans* kindly supplied by Department of Mycology (Animal Health Research Institute, Dokki, Cairo, Egypt). All other chemicals were of analytical or HPLC (High Performance liquid chromatography) grade.

### Chitosanase production

A fungal strain *Dothideomycetes* sp. was used for the production of chitosanase using the fermentation medium consisted of (g/l): chitosan (medium molecular weight), 30;  $K_2HPO_4$ , 1.5;  $MgSO_4$ , 0.4; KCl, 4.0; yeast extract, 18.5 and  $FeSO_4$ , 0.01; at pH 5.5 (Ismail, 2016).

### Partial purification of the enzyme

Partial purification of the produced chitosanase was done by fractional precipitation with ethanol (Thadathil *et al.*, 2016). The fraction with the reasonable specific activity and stability was used in the preparation of COS.

### Chitosanase assay

Chitosanase activity was measured by mixing 500  $\mu$ l of the suitable diluted enzyme solution with 500  $\mu$ l of 1% soluble chitosan (low molecular weight) prepared in 1 M acetic acid and the pH was adjusted to pH 4.5 by using 2 M sodium acetate. The reaction mixture was incubated in shaking water bath at 60°C for 30 minutes. At the end of the reaction time, 2.5 ml of dinitrosalicylic acid was added. The amount of the produced

reducing sugars was estimated immediately using dinitrosalicylic acid method, with D-glucosamine as standard. One international unit of chitosanase was defined as the amount of enzyme that released 1  $\mu$ mol of D-glucosamine per minute under the assay conditions (Ismail, 2016).

### Preparation and analysis of COS

The hydrolysis of chitosan (low molecular weight) was performed using 2% soluble chitosan in 1 M acetic acid (pH 4.5) mixed with chitosanase in a reaction mixture containing enzyme/substrate ratio 0.2 IU/mg incubated for different periods (30 minutes to 6 hours) at 45°C in a shaking water bath. At the end of the incubation period, the reaction was boiled for 10 minutes. The resulted hydrolysate was analyzed by TLC as follows: the chitosan hydrolysate was spotted on a silica gel TLC plate. The plate was run in a mixture of propanol:water:30% ammonia (70:15:15 v/v) as a mobile phase and the amino sugars were visualized with diphenyl amine-aniline reagent (Tanaka *et al.*, 1999). Thin-layer chromatography plates were also used for the separation of COS from the resulted hydrolysate produced by the partial pure enzyme after suitable incubation period and extracted by distilled water after running the plate in the mobile phase then centrifuged for 15 minutes at 5,000 rpm followed by drying then kept until used. The produced mixture of COS was analyzed by HPLC using glucosamine as a standard.

### Extraction of LPS from *Escherichia coli*

LPS was extracted from *Escherichia coli* (O157H7) according to Qureshi *et al.* (1982) by using extraction mixture containing liquid phenol (90 g phenol + 11 ml water), chloroform, and petroleum ether in a volume ratio 2:5:8, respectively.

### Animal and experiment design

Two hundred and fifty Swiss albino mice (25–30 g each) were kept under observation for about 1 week for any signs of disease. Mice in all groups were received balanced ration and plain water *ad libitum*. All experimental procedures involving animals were performed in accordance with the ethical guidelines of the medical ethical committee of the National Research Centre in Egypt.

#### Experiment-1

To study the effect of chitosan (low molecular weight) and COS on the immune status of mice, 100 Swiss albino mice were allocated into five groups (20/each group). Mice in the control group (G1) were injected intraperitoneally (IP) with phosphate buffer saline (PBS), that of G2 and G3 were injected IP with chitosan (10 and 20 mg/kg body weight, respectively), and that G4 and G5 were injected IP with COS (10 and 20 mg/kg body weight, respectively). Chitosan, COS, and PBS were injected every 2 days for 10 days experimental period (five times). Ten mice from each group were kept under observation for 1 week to monitor the mortality rate.

#### Experiment-2

To study the anti-inflammatory effect of chitosan (low molecular weight) and COS on LPS-treated mice, 150 Swiss albino mice were allocated into six groups (25/each group) and

treated as follows: mice in group 1 (A1) were IP injected with PBS and kept as control. Mice in group 2 (A2) were IP injected with LPS (5 mg/kg) in addition to PBS. Mice in group 3 and 4 (A3 and A4) were IP injected with LPS in addition to chitosan in 10 or 20 mg/kg, respectively. While mice in group 5 and 6 (A5 and A6) were IP injected with LPS in addition to COS in 10 or 20 mg/kg, respectively. Chitosan, COS, as well as PBS, was injected three times (one every 2 days) before LPS injection, one time with LPS injection and one-time post-LPS injection by 1 hour. Ten mice from each group were kept under observation up to 1 week to investigate the mortality rate in each group.

### Sampling

#### Peritoneal macrophages

They were collected from mice of all groups (seven samples/ group) to estimate the count of peritoneum cell infiltration (PCI) and phagocytic activities of peritoneal macrophages (PM).

#### Serum samples

They were collected from all groups (seven samples/ group) to estimate the levels of NO, TNF- $\alpha$ , IL-6, and lysozyme activity. The samples were collected once at 10th day (the end of the experiment-1) and twice at 4th hour and 24th hour post-LPS injection (experiment-2).

### Collection, count, and culture of peritoneal macrophage monolayer

Peritoneal cells were obtained according to Victor *et al.* (2003), 10 ml of RPMI medium pH 7.4 was IP injected in each mouse and then peritoneal cells were collected via a needle inserted into inguinal region. After washing the collected cells two times, they were suspended in RPMI medium with 10% FCS, count and adjusted at a density of  $4 \times 10^6$ /ml. The monolayer of PM was obtained by seeding 1 ml of peritoneal cell per cell culture and staining chambers with a cover slip and incubated for 1 hour at 37°C in 5% CO<sub>2</sub> and 90% humidity, after incubation non-adherent cells were removed by washing three times, then reincubated overnight in the same condition.

### Assay of phagocytosis

Phagocytic activity of PM was estimated using *C. albicans* following the method of De-La Fuente *et al.* (2000). The monolayer of macrophage was washed three times then incubated with 1 ml *C. albicans* ( $5 \times 10^6$ /ml RPMI with 15% FCS) for 1 hour at 37°C, 5% CO<sub>2</sub>, and 90% humidity, and washed three time, air-dried, fixed, stained with Giemsa stain and finally, counted 100 PM cell under oil immersion to determine phagocytic percentage (number of macrophages engulf at least one candida spore/100 macrophage cell) and phagocytic index (number of macrophages engulf  $\geq 3$  candida spores/total number of macrophages).

### Estimation of lysozyme activity

Lysozyme activity was measured by agarose gel plate lyses assay according to Peeters and Vantrapen (1977). Briefly, lysoplates were prepared by dissolving 1% agarose in PBS, 0.067 mol/L, pH 6.3, in which *M. lysodeikticus* (50 mg/100 ml agarose) had been dispersed. Nearly, 25  $\mu$ l of serum samples, as well as

the standard lysozyme, were added in each well. After 18 hours, the cleared zones diameter was measured. The concentration of the lysozyme was obtained from logarithmic curve prepared using standard lysozyme solution.

### Estimation of nitric oxide

It carried out according to Yang *et al.* (2010). Briefly, 100  $\mu$ l of serum sample was mixed with 80  $\mu$ l of 375 mM ZnSO<sub>4</sub> and 120  $\mu$ l of 275 mM NaOH, and then centrifuged at 13,000 rpm for 20 minutes to precipitate serum proteins. Supernatant was obtained and added to 400 mg of Cu plated Cd, then shook for 2.5 hours at room temperature after adding 100  $\mu$ l of 0.2 M glycine buffer. Nearly, 100  $\mu$ l supernatant was added into 96 well ELISA plate with 100  $\mu$ l of Griess reagent. The optical density was determined at 545 nm with an ELISA plate reader. Nitric oxide concentration was calculated from the standard curve using NaNO<sub>2</sub>.

### Assay of Tumor necrotic factor-alpha and interleukin-6

Tumor necrotic factor-alpha (TNF- $\alpha$ ) and IL-6 were assayed by using quantikine Elisa kits. (Ray Biotech, Inc) according to the manufacturer's instructions.

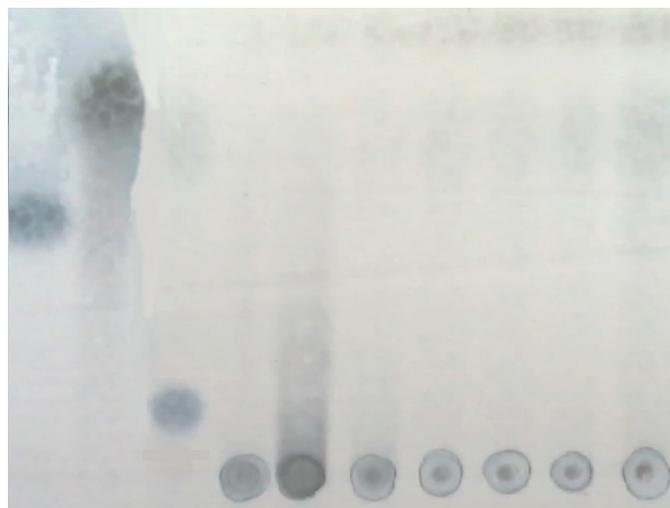
### Statistical analysis

Data were presented as means and standard errors. The significance of the results was evaluated using analysis of variance and LSD using Statistical Package for Social Science.

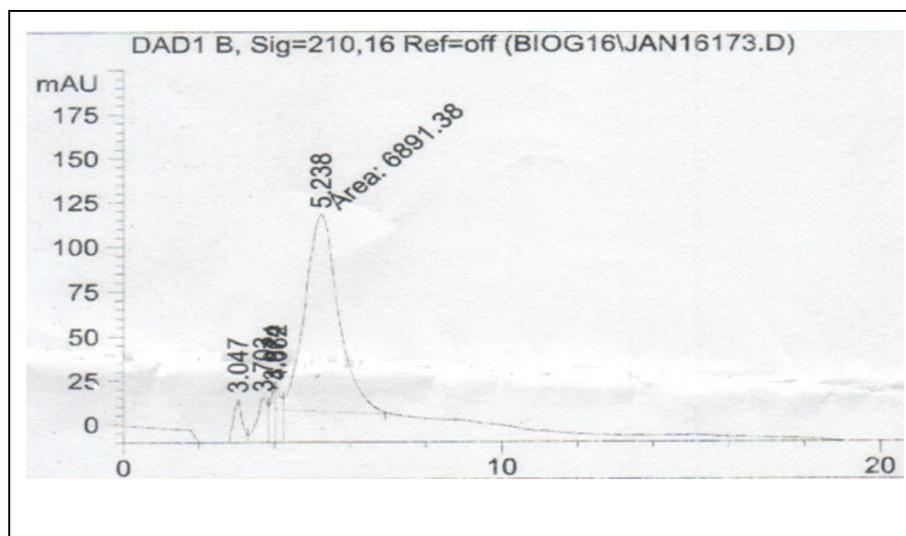
## RESULTS

### Analysis of the hydrolysis product of chitosan

The TLC plate shown in Figure 1 indicated that on the hydrolysis of low molecular weight chitosan by *Dothideomyces* sp. chitosanase, a mixture of COS was produced after 1-hour incubation period. After that, no COS



**Figure 1.** TLC plate of chitoooligosaccharides produced by the hydrolysis of soluble chitosan by *Dothideomyces* sp. chitosanase at various incubation periods. S1: standard glucosamine, S2: standard N-acetyl glucosamine, S3: tetra oligosaccharides.

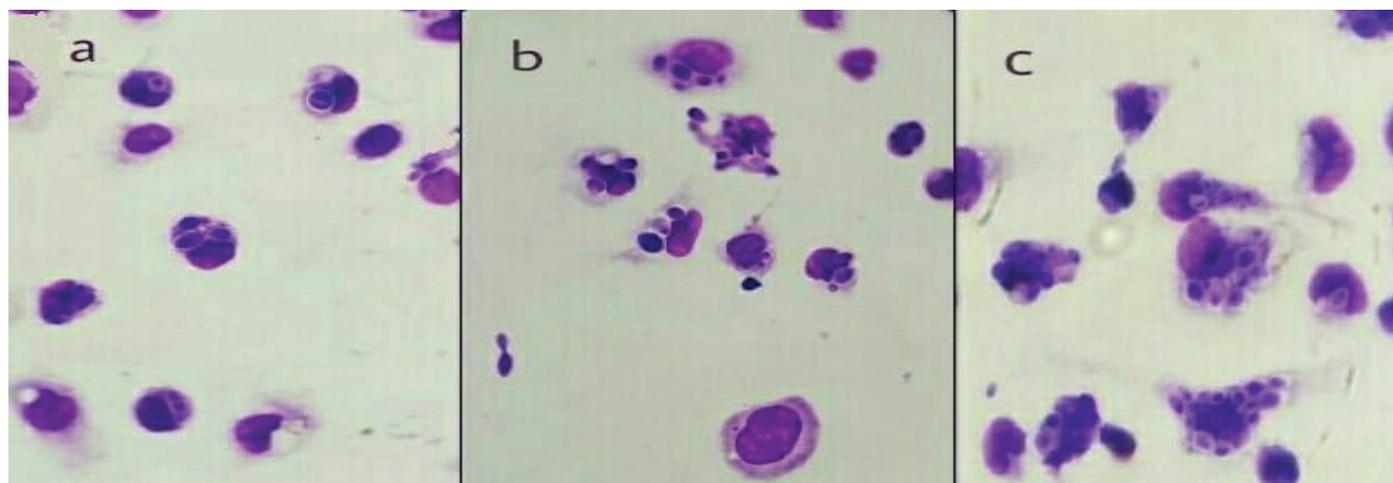


**Figure 2.** HPLC of COS mixture.

**Table 1.** Effect of chitosan and COS injection on the count of peritoneum cell infiltration ( $\times 10^6$ ), phagocytic %, and phagocytic index of peritoneum macrophage of mice.

| Groups<br>Parameters | G1               | G2                   | G3                    | G4                 | G5                 |
|----------------------|------------------|----------------------|-----------------------|--------------------|--------------------|
|                      | Control mice     | Low dose of chitosan | High dose of chitosan | Low dose of COS    | High dose of COS   |
| PCI $\times 10^6$    | 3.1 $\pm$ 0.48A  | 4.3 $\pm$ 0.31B      | 15.6 $\pm$ 2.60abC    | 8.6 $\pm$ 0.52abcD | 13.1 $\pm$ 1.71abd |
| Phagocytic%          | 56.8 $\pm$ 1.55A | 57 $\pm$ 0.71B       | 59.75 $\pm$ 1.11      | 63.3 $\pm$ 3.33 ab | 65.25 $\pm$ 1.89ab |
| Phagocytic index     | 0.42 $\pm$ 0.02A | 0.43 $\pm$ 0.02B     | 0.54 $\pm$ 0.04a      | 0.55 $\pm$ 0.03ab  | 0.56 $\pm$ 0.02ab  |

Values represent means  $\pm$  SE. Mean with small letters indicate significantly different against the capital of the same letters in the same row (between groups) ( $p < 0.05$ ).



**Figure 3.** Mice peritoneal macrophages engulfed or not *C. albicans* spores. (a) mouse peritoneal macrophages of the control group. (b) mouse peritoneal macrophages of group 3, treated with a high dose of Chitosan. (c) mouse peritoneal macrophages of group 5, treated with a high dose of COS. (Giemsa stain  $\times 100$ ).

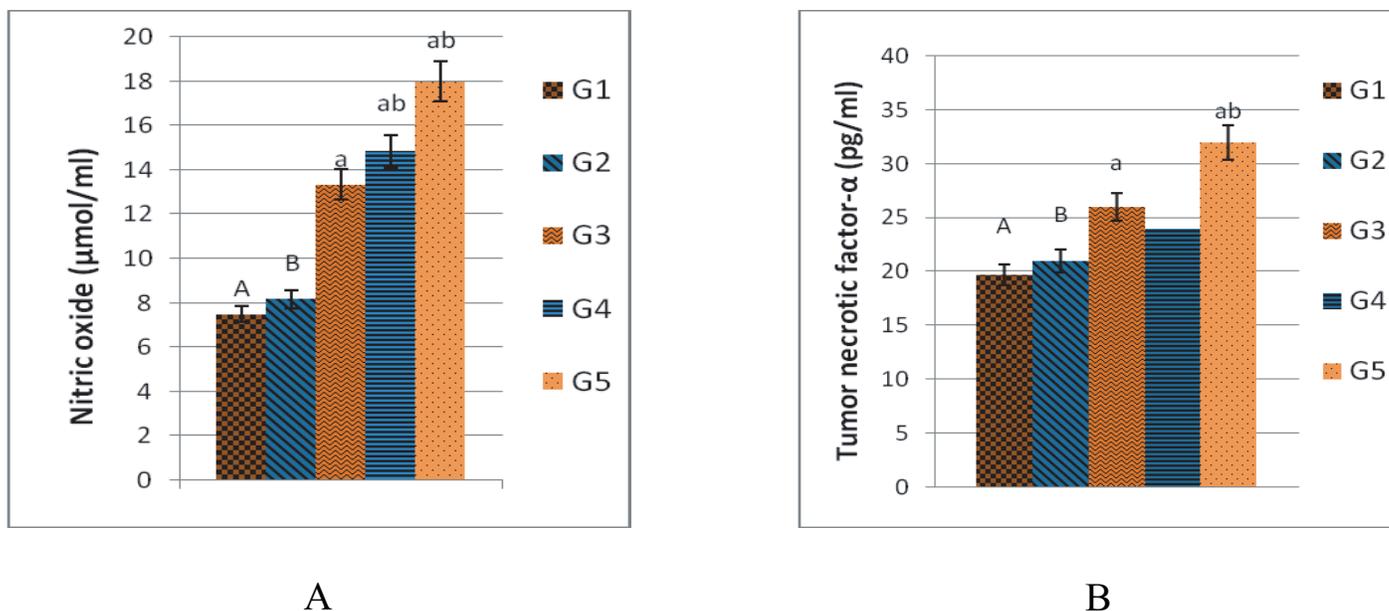
was observed and the monomers were the hydrolysis product. The produced COS mixture was analyzed by HPLC (Fig. 2) compared with the standard glucosamine that appeared at a retention time of 3.061.

### Results of experiment-1

No deaths were recorded in all groups up to 1- week post-chitosan or COS injection.

### *Effect of chitosan and COS injection on peritoneum cell infiltration and phagocytic activity of peritoneal macrophages of mice*

As shown in Table 1 and Figure 3, mice treated with a high dose of chitosan (G3) showed a significant increase in PCI count, phagocytic index of PM comparing with the control group (G1). Also, mice treated with a low and high dose of COS (G4 and G5) recorded a significant increase in PCI count, phagocytic %, and phagocytic index of PM when comparing with mice in both G1 (control group) and G2 (mice treated with low dose of chitosan).



**Figure 4.** Effect of chitosan and COS injection on the level of serum NO ( $\mu\text{mol/ml}$ ) (A) and TNF- $\alpha$  ( $\text{pg/ml}$ ) (B) in mice. G1: control group of mice injected IP with PBS. G2 and G3: groups injected IP with chitosan (10 and 20 mg/kg body weight, respectively). G4 and G5: groups injected IP with COS (10 and 20 mg/kg body weight, respectively). Data are represented as means  $\pm$  SE ( $n = 7$ ). Column with small letters indicates significantly different against the capital of the same letters on the other columns ( $p < 0.05$ ).

**Table 2.** Effect of chitosan and COS injection on IL-6 and lysozyme activity of mice.

| Groups                        | G1                | G2                   | G3                    | G4                 | G5                   |
|-------------------------------|-------------------|----------------------|-----------------------|--------------------|----------------------|
|                               | Control mice      | Low dose of chitosan | High dose of chitosan | Low dose of COS    | High dose of COS     |
| IL-6 ( $\text{pg/ml}$ )       | 159.5 $\pm$ 1.32A | 160.5 $\pm$ 1.19B    | 166 $\pm$ 3.39C       | 161.8 $\pm$ 4.25D  | 179 $\pm$ 2.27abcd   |
| Lysozyme ( $\mu\text{g/ml}$ ) | 149 $\pm$ 4.66A   | 153.25 $\pm$ 6.3B    | 155.3 $\pm$ 3.19C     | 166.8 $\pm$ 3.17Da | 196.5 $\pm$ 5.64abcd |

Values represent means  $\pm$  SE. Mean with small letters indicate significantly different against the capital of the same letter in the same row ( $p < 0.05$ ).

**Table 3.** Effect of chitosan and COS injection on peritoneum cell infiltration ( $\times 10^6$ ) count, Phagocytic% & index of peritoneum macrophage and mortality rate of LPS-treated mice.

| Parameters                  | Time post-LPS injection (hour) | A1                | A2                 | A3                      | A4                       | A5                 | A6                  |
|-----------------------------|--------------------------------|-------------------|--------------------|-------------------------|--------------------------|--------------------|---------------------|
|                             |                                | Control           | LPS                | LPS + Chitosan low dose | LPS + Chitosan high dose | LPS + COS low dose | LPS + COS high dose |
| PCI ( $\times 10^6$ )       | 4                              | 3.4 $\pm$ 0.24A   | 36.5 $\pm$ 2.45a   | 35.6 $\pm$ 1.5a         | 33.4 $\pm$ 2.25a         | 35.2 $\pm$ 1.11a   | 37.5 $\pm$ 3.12a    |
|                             | 24                             | 3.5 $\pm$ 0.31A   | 24 $\pm$ 1.89aB    | 25 $\pm$ 3.50ac         | 23 $\pm$ 3.57a           | 18.8 $\pm$ 2.58abc | 15 $\pm$ 2.2abc     |
| Phagocytic %                | 4                              | 57.75 $\pm$ 1.25A | 73.25 $\pm$ 1.55Ba | 69.75 $\pm$ 1.89Ca      | 69.75 $\pm$ 1.44Dac      | 71.5 $\pm$ 1.32Ead | 60.25 $\pm$ 1.93be  |
|                             | 24                             | 60 $\pm$ 1.29A    | 71.75 $\pm$ 2.46Ba | 67.5 $\pm$ 1.85Ca       | 65.75 $\pm$ 1.25ab       | 61.5 $\pm$ 2.63bc  | 58.3 $\pm$ 2.32bc   |
| Phagocytic index            | 4                              | 0.44 $\pm$ 0.23A  | 0.64 $\pm$ 0.02Ba  | 0.62 $\pm$ 0.02Ca       | 0.59 $\pm$ 0.02Dea       | 0.60 $\pm$ 0.01Ea  | 0.49 $\pm$ 0.02bee  |
|                             | 24                             | 0.45 $\pm$ 0.03A  | 0.60 $\pm$ 0.03Ba  | 0.59 $\pm$ 0.03Ca       | 0.54 $\pm$ 0.04Dab       | 0.51 $\pm$ 0.04ab  | 0.47 $\pm$ 0.04bcd  |
| Mortality rate up to 1 week |                                | –                 | 90%                | 80%                     | 70%                      | 60%                | 40%                 |

Values represent means  $\pm$  SE. Mean with small letters indicate significantly different against the capital of the same letter in the same row ( $p < 0.05$ ).

#### Effect of chitosan and COS injection on NO and TNF- $\alpha$ in mice

Mice treated with high dose of chitosan or COS (G3 and G5) showed a significant increase in NO and TNF- $\alpha$  compared with the control group (G1). Also, mice treated with low dose of COS (G4) showed a significant increase in NO (Fig. 4).

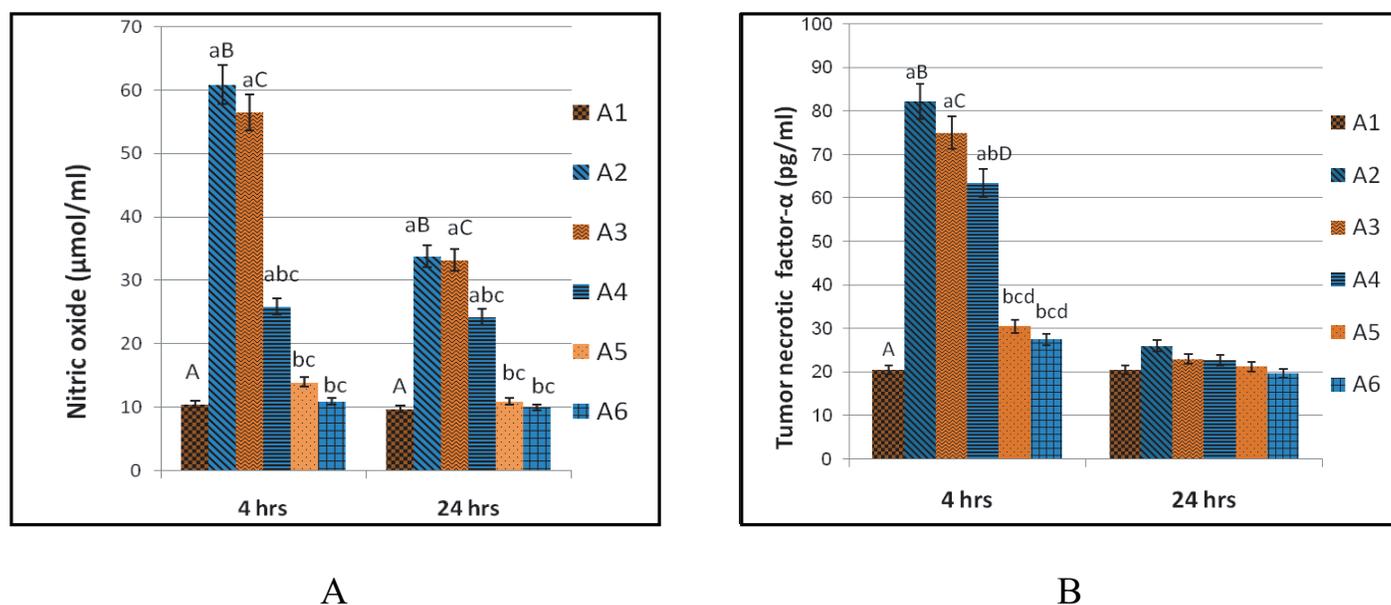
#### Effect of chitosan and COS injection on interleukin-6 and lysozyme activity

Mice treated with high dose of COS (G5) showed a significant increase in IL-6 value and lysozyme activity comparing with control and all other treated groups. Also, mice treated with

low dose of COS (G4) showed a significant increase in lysozyme activity comparing with the control group (Table 2).

#### Results of experiment-2

The mortality rate in LPS group was 90% of mice which died within 48 hours, while mice pretreated with chitosan and COS at low and high dose partially protect mice from LPS induced mortality (Table 3), the best protection recorded with a high dose of COS. The mice in the LPS group (A2) gave obvious significant increasing in all tested parameters.



**Figure 5.** The effect of chitosan and COS on the level of serum NO ( $\mu\text{mol/ml}$ ) (A) and TNF- $\alpha$  ( $\text{pg/ml}$ ) (B) of LPS-treated mice. A1: mice of the control group injected IP with PBS. A2: group injected IP with LPS (5 mg/kg). A3 and A4: groups injected IP with LPS in addition to chitosan (10 or 20 mg/kg, respectively). A5 and A6: groups injected IP with LPS in addition to COS (10 or 20 mg/kg, respectively). Data are represented as means  $\pm$  SE ( $n = 7$ ). Column with small letters indicates significantly different against the capital of the same letters on the other columns ( $p < 0.05$ ) in each time.

#### Effect of chitosan and COS injection on peritoneum cell infiltration and phagocytic activity of peritoneal macrophage of LPS-treated mice

Treatment of mice with a high dose of chitosan (A4) revealed a significant decrease in phagocytic % and index after 24-hour post-LPS injection comparing with LPS group, but it still significantly increased compared to the control group. Mice treated with COS at low dose (A5) recorded a significant decrease in the count of PCI, phagocytic %, and index of PM at 24th hour post-LPS injection comparing with LPS group, however, COS at high dose (group A6) significantly decreased the count of PCI at 24th hour post-LPS injection and significantly decreased the phagocytic % and index at 4th hour and 24th hour post-LPS injection to approach the control values.

#### The effect of chitosan and COS injection on the levels of nitric oxide and TNF- $\alpha$ of LPS-treated mice

Treatment of mice with a high dose of chitosan (A4) revealed significant decrease in NO values at 4th and 24th hour post-LPS injection and in TNF- $\alpha$  at 4th hour post-LPS injection comparing to LPS group but it still significantly increased compared to the control group. However, COS at a low and high dose (A5 and A6) significantly decreased NO values at 4th and

24th hour post-LPS injection and TNF- $\alpha$  at 4th hour post-LPS injection to approach the control values. TNF- $\alpha$  retained to basal value in all groups at 24th hour post-LPS injection (Fig. 5).

#### The effect of chitosan and COS on the levels of interleukin-6 and lysozyme activity on LPS-treated mice

Treatment of mice with a high dose of chitosan (A4) revealed a significant decrease in IL-6 at 24 hours and in lysozyme at 4th and 24th hour post-LPS injection in comparing with LPS group. Also, treatment of mice with COS at a low and high dose (A5 and A6) showed significant decrease in the levels of IL-6 and lysozyme at 4th and 24th hour post-LPS injection to approach the control values (Table 4).

## DISCUSSION

Natural products had received much attention to modulate the immune system since the current treatments induce severe side effects (Mei *et al.*, 2013; Yang *et al.*, 2016). Several natural compounds as polysaccharides and oligosaccharides reported to enhance the immune system (Petrovsky *et al.*, 2011). Chitosan and COS have gained a great attention due to their significant *in vitro* immunomodulatory effects (Davydova *et al.*, 2016; Zheng *et al.*, 2018).

**Table 4.** The effect of chitosan and COS on the levels of Interleukin-6 and lysozyme activity on LPS-treated mice.

|                               | Time post-LPS injection (hour) | A1              | A2                | A3                    | A4                     | A5                | A6                 |
|-------------------------------|--------------------------------|-----------------|-------------------|-----------------------|------------------------|-------------------|--------------------|
|                               |                                | Control         | LPS               | LPS+Chitosan low dose | LPS+Chitosan high dose | LPS+COS low dose  | LPS+COS high dose  |
| IL-6 ( $\text{pg/ml}$ )       | 4                              | 164 $\pm$ 2.66A | 203 $\pm$ 4.96Ba  | 201 $\pm$ 4.69Ca      | 196 $\pm$ 3.7aD        | 181 $\pm$ 5.72bE  | 176 $\pm$ 4.81bcde |
|                               | 24                             | 169 $\pm$ 2.95A | 260 $\pm$ 8.44Ba  | 240 $\pm$ 3.5Ca       | 208 $\pm$ 5.18Dab      | 196 $\pm$ 4.61Ebc | 174 $\pm$ 5.59bcde |
| Lysozyme ( $\mu\text{g/ml}$ ) | 4                              | 173 $\pm$ 6.14A | 465 $\pm$ 11.25Ba | 376 $\pm$ 6.48Ca      | 213 $\pm$ 7.58Dabc     | 218 $\pm$ 8.14Ebd | 186 $\pm$ 8.64bcde |
|                               | 24                             | 180 $\pm$ 6.49A | 320 $\pm$ 31.4Ba  | 291 $\pm$ 10.5Ca      | 231 $\pm$ 9.78ab       | 203 $\pm$ 7.79b   | 180 $\pm$ 7.01bc   |

Values represent means  $\pm$  SE. Mean with small letters indicate significantly different against the capital of the same letter in the same row ( $p < 0.05$ ).

The *in vivo* immunomodulatory effects of chitosan and COS were studied in two experiments as follows: Experiment-1: the immune-stimulatory effect of chitosan and COS on normal mice; Experiment-2: the anti-inflammatory effect of chitosan and COS in LPS-treated mice.

The obtained results of experiment-1 revealed that chitosan or COS in low and high doses (10 and 20 mg/kg) were non-toxic as no mortality was observed. This result agrees with Davydov *et al.* (2016), Kim *et al.* (2014), and Santos-Moriano *et al.* (2018). Intraperitoneal injection of a high dose of chitosan caused a significant increase of PCI, phagocytic index of PM and increase in the serum level of TNF- $\alpha$  and NO, while the immune-stimulatory effect of COS was more pronounced as COS in low and high doses showed a significant increase in nearly all tested parameters. The effect of chitosan is supported by previous studies which recorded that low or high molecular weight of Chitosan caused an increase of peritoneum cell count in the lavages (Brodaczewska and Doligalska, 2013) and significantly enhance the pinocytic activity and the production of TNF- $\alpha$  and NO in RAW264.7 macrophages (Wu *et al.*, 2015). Also, Davydov *et al.* (2016) reported that chitosan (5200Da) tripled the synthesis of TNF- $\alpha$  in human blood cells. The immune-stimulatory effect of chitosan may be attributed to the similarity in their structure and the saccharide portion of lipid A in LPS and so it may similarly bind to the surface receptors of CD-14 or TLR-4 of the PM to activate the signal transduction system (Wu *et al.*, 2007). In contrary, Guzman-Morales *et al.* (2011) found that chitosan-stimulated murine bone marrow-derived macrophages failed to produce NO. These discrepancies could be potentially explained by the difference in dosages, molecular weight, and the degree of acetylation of chitosan used in each study.

The obvious immune-stimulatory effect of COS recorded in this study was supported by several studies which showed that COS cause a significant increase in the levels of TNF- $\alpha$  (Feng *et al.*, 2004) and NO (Wu *et al.*, 2007). Also, it improved the immunity of broilers by the release of IL-1, IL-6, TNF- $\alpha$ , and NO (Deng *et al.*, 2008). The immune-stimulatory effect of COS may be due to its internalization to macrophage through a macrophage lectin receptor with mannose specificity (Feng *et al.*, 2004) or due to binding with CD14 receptor and TLR-4 of macrophage which lead to activation of nuclear factor kappa beta (NF- $\kappa$ B) and activation of genes expression (Wu *et al.*, 2007).

The anti-inflammatory effects of chitosan and COS in LPS-elicited inflammatory response in mice were determined. Intraperitoneal injection of LPS alone (5 mg/Kg) induces a potent inflammatory response in mice, manifested by a marked increase in all tested parameters. These results come in accordance with Kim *et al.* (2014) and Yang *et al.* (2016). It was known that LPS is recognized by TLR-4 on immune cells which activate (NF- $\kappa$ B) through stimulation of I $\kappa$ B kinase which phosphorylates I $\kappa$ B and thereby induces its degradation resulting in dissociation and translocation of NF- $\kappa$ B into the nucleus and activation of the target genes of inflammatory cytokines (Li *et al.*, 2014).

Both chitosan and COS had different protecting effects against LPS-induced mortality in mice. The less mortality rate (40%) was recorded in the group injected with a high dose of COS (A6), while that of LPS group was 90%. IP injection with a high

dose of Chitosan and both low and high dose of COS significantly overcome LPS-induced severe activation of PM and modulate the overproduction of NO, lysozyme, TNF- $\alpha$ , and IL-6, which approach the control value as in COS groups or not as in chitosan groups. The down-regulation of LPS-induced inflammation by chitosan come in accordance with (Ji *et al.*, 2013; Yermak *et al.*, 2006) which attributed to the formation of a complex with LPS by ionic binding between negatively charged groups on LPS (anionic compound) and positively charged amino groups on chitosan (cationic polymer) (Wilkinson *et al.*, 1996). These LPS-chitosan complexes are accompanied by the essential modification of several immunological properties of LPS and possess 10 to 20 times less toxicity than LPS alone (Yermak *et al.*, 2006), which may be attributed to the blocking of the toxophoric center of endotoxin or the alterations in the molecular charge and/or the structure of LPS (Ji *et al.*, 2013).

The down-regulation of LPS-induced inflammation by COS agreed with many *in vitro* studies which reported that COS exposure led to dose-dependent attenuation of LPS induced secretion of NO, TNF- $\alpha$ , and IL-6 in RAW264.7 cells (Kim *et al.*, 2014; Xu *et al.*, 2017; Yoon *et al.*, 2007) and in microglia cell (Pangestuti *et al.*, 2011; Wei *et al.*, 2012). This anti-inflammatory feature of COS may be attributed to its reduction of gene expression of inflammatory cytokines (Deng *et al.*, 2008) and suppression of the translocation of a subunit of NF- $\kappa$ B into the nucleus by inhibiting degradation of I $\kappa$ B- $\alpha$  p65 (Kim *et al.*, 2014). Also, Liu *et al.* (2010) suggested that COS inhibit LPS induced up-regulation of IL-6 by at least two parallel signaling pathways: one via p38 mitogen-activated protein kinases pathway and one via the extracellular signal-regulated kinase 1/2 pathway. From the above data, it was recorded that both chitosan and COS have dual activities, both immune-stimulatory activity in normal mice model and anti-inflammatory activity in LPS-inflamed mice model. It was explained by the fact that once the inflammatory cascade is induced, other mechanisms operate to down-regulate the process of inflammation. In the same regard, Porporatto *et al.* (2003) recorded that high molecular weight chitosan interacting with resident macrophages by stimulated NO production and with inflammatory macrophages by inhibition of NO production through enhanced the arginase expression.

The more pronounced activities of COS present in this study may be attributed to their highly water solubility and lower viscosity compared with the parent chitosan that was characterized by its highly viscous nature with lower water solubility that lead to a less efficient interaction with the cell (Liaqat and Eltem, 2018; Sanchez *et al.*, 2017).

In conclusion, the produced mixture of COS possessed an immunostimulatory activity in normal mice as that of the parent chitosan; therefore, both might be used as functional food components and additives. Moreover, they have an anti-inflammatory effect on LPS-treated mice that potentially enable their use as therapeutic agents for disorders where inflammation is one of the pathological features. Chito-oligosaccharide is clearly much better than chitosan as an immunomodulatory agent.

#### CONFLICT OF INTEREST

Natural product and their biological applications.

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