RESEARCH ARTICLE

Mycobiology 40(4) : 236-243 (2012)
http://dx.doi.org/10.5941/MYCO.2012.40.4.236
pISSN 1229-8093
eISSN 2092-9323

Anticancer and Immunopotentiating Activities of Crude Polysaccharides from *Pleurotus nebrodensis* on Mouse Sarcoma 180

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(Received November 16, 2012. Revised November 23, 2012. Accepted December 3, 2012)

***Pleurotus nebrodensis* is an edible and commercially available mushroom in Korea. This study was conducted in order to evaluate the anticancer and immunopotentiating activities of crude polysaccharides, extracted in methanol, neutral saline, and hot water (hereafter referred to as Fr. MeOH, Fr. NaCl, and Fr. HW, respectively) from the fruiting bodies of *P. nebrodensis*. β-Glucan and protein contents in Fr. MeOH, Fr. NaCl, and Fr. HW extracts of *P. nebrodensis* ranged from 23.79~36.63 g/100 g and 4.45~6.12 g/100 g, respectively. Crude polysaccharides were not cytotoxic against sarcoma 180, HT-29, NIH3T3, and RAW 264.7 cell lines at a range of 10~2,000 μg/mL. Intraperitoneal injection with crude polysaccharides resulted in a life prolongation effect of 11.76~27.06% in mice previously inoculated with sarcoma 180. Treatment with Fr. NaCl resulted in an increase in the numbers of spleen cells by 1.49 fold at the concentration of 50 μg/mL, compared with control. Fr. HW improved the immuno-potentiating activity of B lymphocytes through an increase in alkaline phosphatase activity by 1.65 fold, compared with control at 200 μg/mL. Maximum production of nitric oxide (14.3 μM) was recorded in the Fr. NaCl fraction at 200 μg/mL. Production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) was significantly higher, compared to control, and IL-6 production was highest, in contrast to TNF-α, IL-1β, and positive control, concanavalin at the tested concentration of the various fractions. Results of the current study suggest that polysaccharides extracted from *P. nebrodensis* have a strong anticancer effect and may be useful as an ingredient of biopharmaceutical products for treatment of cancer.**

KEYWORDS : Anticancer activity, Crude polysaccharides, Immunomodulation, Mouse sarcoma 180, *Pleurotus nebrodensis*

Introduction

The biological activities of polysaccharides have attracted increasing attention in biochemistry and medicine. The antitumor activities of polysaccharides have been reported to result primarily from their immunopotentiation effects [1, 2]. Polysaccharides can stimulate immune cells, such as granulocytes, monocytes, macrophages, and nature killer cells to trigger secretion of cytokines that will stimulate the immune system [3]. Chemical modification has been reported to enhance the immunopotentiation and antitumor activities of polysaccharides, due to the change of substituent groups and chain conformation of polysaccharides [4]. Polysaccharides can interact with receptors of immune cells to trigger immunological responses, including antitumor activity [5].

A large number of chemical compounds identified as specific agents for killing cancer cells are also toxic to normal cells. Many potential anticancer drugs have considerable side effects. Therefore, discovery of new safer drugs with activity against tumor has become an important goal of research in biomedical sciences. The potentiation of host defense mechanisms has emerged as a possible means of inhibiting tumor growth without causing harm to the host. From this of point of view, extensive studies have been conducted for investigation of polysaccharides extracted from mushrooms.

Pleurotus nebrodensis, known as Bailingu oyster

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mushroom [6], is a good source of dietary fiber and valuable nutrients It also contains a number of biologically active compounds with therapeutic activities, such as modulation of the immune system, and inhibition of tumor growth [7, 8]. Regardless of the clinical importance and the therapeutic potential of *P. nebrodensis*, no studies on its anticancer and immunopotentiating activities have been reported. The goal of the current study is to evaluate the immunostimulating and antitumor effects of crude polysaccharides extracted from the fruiting bodies of *P. nebrodensis* on mouse sarcoma 180. The *in vivo* antitumor effect in sarcoma 180 tumor bearing mice and *in vitro* cytotoxic activities of four cell lines were studied. In addition, proliferation of murine spleen cells, alkaline phosphatase (APase) activity, nitric oxide (NO), and cytokine production in murine peritoneal macrophages were also investigated.

Materials and Methods

This study was conducted from November 2010 to December 2011 at the Animal House and Laboratory of Applied Microbiology, Division of Life Sciences, University of Incheon, Korea, and the experimental protocols were approved by the Animal Care Ethics Committee of the University, Korea. All experimental procedures were performed in accordance with the guide for the care and use of experimental animals.

Mushroom and extraction. Fresh fruiting bodies of *P. nebrodensis* were obtained from the Mushroom Research Institute of Gyeonggi Province in Korea. A pure culture was deposited in the Culture Collection and DNA Bank of Mushroom (CCDBM), Division of Life Sciences, University of Incheon, Korea, and an accession number was acquired, IUM-4658. Fresh fruiting bodies were dried with hot air at 40°C for 48 hr and pulverized.

Two hundred grams of pulverized fruiting bodies of *P. nebrodensis* were extracted with 3,000 mL of 80% methanol and neutral saline (0.9% NaCl) with stirring at 150 rpm for 24 hr at 25°C to obtain methanolic and NaCl extracts. The mixture was filtered through two layers of Whatman No. 1 filter paper (Whatman, Maidstone, UK). The same quantity of sample was boiled at 100°C for 3 hr with 3,000 mL deionized distilled water to obtain a hot water extract. The mixture was cooled at room temperature and filtered through a Whatman No. 1 filter paper. The residues of methanol, NaCl, and hot water extraction were then treated two more times in the same manner. All supernatants obtained of each extract were combined and mixed with four volumes of ethanol and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation, dissolved in distilled water, dialyzed for 48 hr at 4°C, and lyophilized. These fraction were referred to as the methanol extract (Fr. MeOH), neutral saline extract (Fr. NaCl), and hot water extract (Fr. HW). The yields from the methanolic, NaCl, and hot water extracts of *P. nebrodensis* were 19.04, 15.44, and 17.84% (w/w), respectively.

Animals. Five-wk-old inbred male ICR mice (20 ± 2 g) were purchased from Central Lab Animal Inc. (Seoul, Korea). All mice were acclimated to the animal house for a period of one week. Mice were housed in an animal room at 23 ± 2°C under 12 hr dark-light cycles (17:00~05:00 hr) and a relative humidity of 50~60%. During the experimental period, mice received the standard basal diet, purchased from Central Lab Animal Inc.

Cell lines. Mouse sarcoma 180, colon cancer (HT-29), mouse embryonic fibroblast cells (NIH3T3), and murine macrophage cell (RAW264.7) lines were purchased from the Korean Cell Line Bank of Seoul National University (Seoul, Korea). HT-29, NIH3T3, and RAW264.7 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum (FBS) at 37°C with 5% atmospheric CO_2 in a humidified incubator. Sarcoma 180 cells were maintained in ascitic form by serial transplantation every seven days in a male ICR mouse.

Determination of β-glucan and total protein. The β-glucan contents of three different fractions extracted from the fruiting bodies of *P. nebrodensis* were quantitatively determined using a mushroom and yeast β-glucan assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). In brief, for determination of total glucan (α- and β-), 5 mg of each fraction was suspended in 75 μL of concentrated HCl and incubated at 45°C for 30 min, followed by addition of 500 μL distilled water, and placed in a boiling water bath for 2 hr. The pH was neutralized with 500 μL of 2 N KOH, and centrifuged for 10 min at 1,500 ×g. Fifty microliters of the supernatant was digested with aliquots of exo-1,3-β-glucanase (20 U/mL) plus β-glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0). The hydrolysates were incubated with a mixture of 1.5 mL of glucose oxidase/peroxidase (GO/POD) and incubated at 40°C for 1 hr. The absorbance of the solution was measured at 510 nm. For measurement of α-glucan, 5 mg of each fraction was suspended in 100 μL of 2 N KOH for 20 min, followed by neutralization with 400 μL of 1.2 M sodium acetate buffer (pH 3.8). Then, the solution was centrifuged for 10 min at 1,500 ×g and aliquots of amyloglucosidase (1,630 U/mL) plus invertase (500 U/mL) were added to 50 μL of supernatant and incubated at 40°C for 30 min. The solution was incubated with 1.5 mL of a mixture of GO/POD at 40°C for 20 min and absorbance was measured at 510 nm. β-Glucan was

determined by subtracting α-glucan from total glucan content.

Protein content of each fraction was quantified by the Bradford method [9], using bovine serum albumin (BSA) as a standard. Total protein content of the fractions is expressed as g of BSA equivalent per 100 g of dry weight.

Cytotoxicity. A rapid colorimetric method previously described by Mosmann [10] was used in the 3-(4,5-dimethyl-1-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, for measurement of cell viability and proliferation. Briefly, for the MTT assay, 100 μL of cells of HT-29, NIH3T3, and RAW 264.7 (1×10^5 cells/well) were treated with 10, 100, 1,000, and 2,000 μg/mL concentrations of three different fractions (Fr. MeOH, Fr. NaCl, and Fr. HW) of *P. nebrodensis* and cultured for 24 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Thereafter, 10 μL of 5 mg/mL of MTT solution was added, followed by incubation at 37°C with 5% atmospheric CO_2 for 4 hr under dark conditions. Following removal of the supernatant, purple formazan crystals produced were dissolved in 100 μL of dimethylsulfoxide, and quantified by measurement of optical density (OD) at 570 nm using a microplate reader. For the cytotoxicity assay of sarcoma 180, 50 μL of sarcoma 180 cells (2×10^5 cells/well) were treated with 10, 100, 1,000, and 2,000 μg/mL concentrations of three different fractions of *P. nebrodensis* and cultured for 24 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Then, 1 mg/mL of 2,3-bis(2-methoxyl-4nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) solution was mixed with 30 μL of 25 μM phenazine methosulfate, followed by incubation at 37°C with 5% atmospheric CO_2 for 2 hr under dark conditions. OD was then measured at 450 nm using a microplate reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells that served as control.

***In vivo* assay of antitumor activity.** Antitumor activities of three different fractions of *P. nebrodensis* were assayed against mouse sarcoma 180 cells (ascitic type, 5×10^5 cells) implanted in a six-wk-old ICR mouse. The test sample was dissolved in phosphate buffered saline (PBS, pH 7.4; Gibco BRL, Gaitherburg, MD, USA) and filtered through a 0.22 μm membrane filter (Millipore Co., Bedford, MA, USA), followed by intraperitoneal injection in mice for 10 consecutive days at a dose of 20 mg/kg, starting from 24 hr after tumor implantation. Antitumor activities of *P. nebrodensis* against sarcoma 180 tumor bearing ICR mice were evaluated according to the increase in life span (ILS). The method previously described by Geran *et al*. [11] was used for calculation of ILS.

$$\text{ILS}\ (\%) = [(T - C)/C)] \times 100$$

Where, T is the mean of survival days (MSD) of the treated groups and C is the MSD of the control group.

Proliferation of murine spleen cells. The WST-1 assay was performed to test for proliferation of murine spleen cells [12]. Six-wk-old male ICR mice were sacrificed by cervical dislocation, followed by aseptic removal of the spleen and grinding of the spleen using a 100-mesh sieve (Bellco Glass Inc., Vineland, NJ, USA). Two volumes of lymphocyte separation medium (PAA Laboratory Gmbh, Pasching, Austria) were added to the extracted solution, which was then centrifuged for 20 min at 400 ×g. Monocyte cells of spleen were selectively separated and centrifuged three times for approximately 5 min at 300 ×g. The spleen cells (2×10^5 cells/mL) were then added to RPMI 1640 medium supplemented with heat inactivated FBS, followed by treatment with 50, 200, and 500 μg/mL concentrations of the three different extracts of *P. nebrodensis* and incubated for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2 under dark conditions. In the same manner, as a positive control, cells were treated with lipopolysaccharide (LPS) at concentrations of 5 and 10 μg/mL. Thereafter, 10 μL of a 5 mg/mL concentration of WST-1 assay solution was added to each well, followed by incubation for 4 hr at 37°C with 5% atmospheric CO_2 under dark conditions. OD was measured at 440 nm using a microplate reader.

APase activity in murine spleen cells. A method previously described by Ohno *et al*. [13] was used for measurement of APase activity of murine spleen cells. Six-wk-old male ICR mice were sacrificed by cervical dislocation and cell suspension of the spleen was prepared aseptically. Fifty, 100, and 200 μg/mL concentrations of the three different extracts of *P. nebrodensis* were applied to 100 μL of spleen cells (1×10^6 cells/well), followed by incubation for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. LPS at 5 and 50 μg/mL was applied to 100 μL of spleen cells (1×10^6 cells/well), followed by incubation for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Cell suspensions were collected and freeze-thawed, followed by addition of 50 mM of sodium carbonate buffer (pH 9.8) containing *p*-nitrophenyl-phosphate (0.1 mg/mL) and $MgCl_2$ (1 mM) to 10 μL of the cell lysate. The reaction mixture was incubated for 1 hr at 37°C with 5% atmospheric CO_2 and was terminated by addition of 500 μL of 0.3 N ice cold NaOH. Absorbance was measured at 405 nm. APase activity of spleen cells was expressed as the stimulation index (SI).

$$\text{SI} = \text{mean OD in the treated group} / \text{mean OD in control group.}$$

NO production by RAW 264.7 macrophages. The method previously described by Choi *et al*. [14] was used for assessment of NO production in the culture supernatants of RAW 264.7. Briefly, 100 μL of RAW 264.7 cells (1 ×

10^5 cells/well) were treated with 50, 100, and 200 μg/mL concentrations of three different extracts of *P. nebrodensis* and incubated for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. LPS, as the positive control, was applied to 100 μL of RAW 264.7 cells (1×10^5 cells/well) at 1, 10, and 50 μg/mL, followed by culture for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO_2. Then, an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid) was mixed with the culture supernatants and allowed to stand for 10 min. OD was measured at 540 nm using a microplate reader. Nitrite concentration was calculated from a standard curve prepared with known concentrations of sodium nitrite.

Determination of cytokine production in murine peritoneal macrophages. Six-wk-old male ICR mice were sacrificed by cervical dislocation, followed by washing the peritoneal cavity with 5.0 mL of sterile cold PBS, and passed through a 100-mesh sieve for removal of debris. Then, the exudate cells were centrifuged at 400 ×g for 30 min and pelleted cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg streptomycin at 37°C with 5% atmospheric CO_2 for 1 hr. Nonadherent cells were removed with warm PBS and adherent cells were then trypsinized and viable cell counts (Trypan blue test) were performed using a haemacytometer. Macrophages at a density of 5×10^5 cells/mL were cultured with three different concentrations (10, 100, and 1,000 μg/mL) of each extract and incubated for 48 hr in 24-well microplates at 37°C with 5% atmospheric CO_2 under dark conditions. In the same manner, positive controls, LPS and concanavalin (Con A), were incubated with different concentrations of 1, 5, and 10 μg/mL; supernatants were collected and used for the enzyme-linked immunosorbet assay (ELISA) cytokine assay. Cytokine levels were measured using a commercially available ELISA kit (KOMA Biotech, Seoul, Korea) for tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) according to the manufacturer's instruction. Standard curves were used for calculation of cytokine concentration.

Statistical analysis. Data were expressed as mean ± SD. Intergroup differences were analyzed by a one-way analysis of variance followed by Duncan's new multiple-range test. SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA) was used for the analysis. A $p \leq 0.05$ was considered statistically significant.

Results

β-Glucan and protein content. β-Glucan and protein contents of various fractions, extracted from the fruiting bodies of *P. nebrodensis* were measured (Table 1). The highest amount of β-glucan was recorded in Fr. MeOH (36.63 g/100 g), followed by Fr. HW (29.37 g/100 g) and Fr. NaCl (23.79 g/100 g), respectively. The protein level in Fr. NaCl was significantly higher ($p \leq 0.05$), compared to Fr. MeOH and Fr. HW.

Table 1. β-Glucan and protein contents of various fractions extracted from the fruiting bodies of *Pleurotus nebrodensis*

Fractions	β-Glucan	Protein
Fr. MeOH	36.63 ± 1.12^a	4.67 ± 0.56^b
Fr. NaCl	23.79 ± 1.59^c	6.12 ± 0.26^a
Fr. HW	29.37 ± 2.58^b	4.45 ± 1.21^b

Values are expressed as mean ± SD (n = 3). Values in the second and third column that do not share a common superscript are significantly different at $p \leq 0.05$.
Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl, fractions extracted with 0.9% NaCl solution; Fr. HW, fractions extracted with hot water.

Cytotoxic activity. Results for *in vitro* cytotoxic activities of three different fractions (Fr. MeOH, Fr. NaCl, and Fr. HW) of *P. nebrodensis* against sarcoma 180, HT-29, NIH3T3, and RAW 264.7 are shown in Fig. 1. Maximum cell viabilities on sarcoma 180, HT-29, NIH3T3, and RAW 264.7 of Fr. MeOH, Fr. NaCl, and Fr. HW fractions were recorded at the concentration of 10 μg/mL. At 10~2,000 μg/mL, cell viability of Fr. MeOH, Fr. NaCl, and Fr. HW fractions against sarcoma 180, HT-29, NIH3T3, and RAW 264.7 cell lines ranged from 120~127, 67~116, and 69~100%, respectively (Fig. 1A), 94~101, 68~103, and 75~103%, respectively (Fig. 1B), 98~115, 75~106, and 85~109%, respectively (Fig. 1C), and 98~114, 67~100, and 103~116%, respectively (Fig. 1D).

***In vivo* antitumor activity of fractions.** Antitumor activities of Fr. MeOH, Fr. NaCl, and Fr. HW fractions from the fruiting bodies of *P. nebrodensis* were tested against sarcoma 180 tumor bearing mice. According to the results, the greatest increase in life span was recorded in Fr. NaCl (27.06%), followed by Fr. HW (21.76%) and MeOH (11.76%), respectively, compared to the control group (Fig. 2).

Effect of fractions on proliferation of murine cells. The effect of Fr. MeOH, Fr. NaCl, and Fr. HW fractions of *P. nebrodensis* on proliferation of murine cells is shown in Fig. 3. The results indicated that Fr. NaCl showed significantly good activity, while Fr. HW and Fr. MeOH showed moderate activity, compared to control. However, at the concentration of 50 μg/mL LPS showed excellent activity.

APase activity in murine spleen cells. Stimulation of splenic lymphocytes with LPS, Fr. MeOH, Fr. NaCl, and

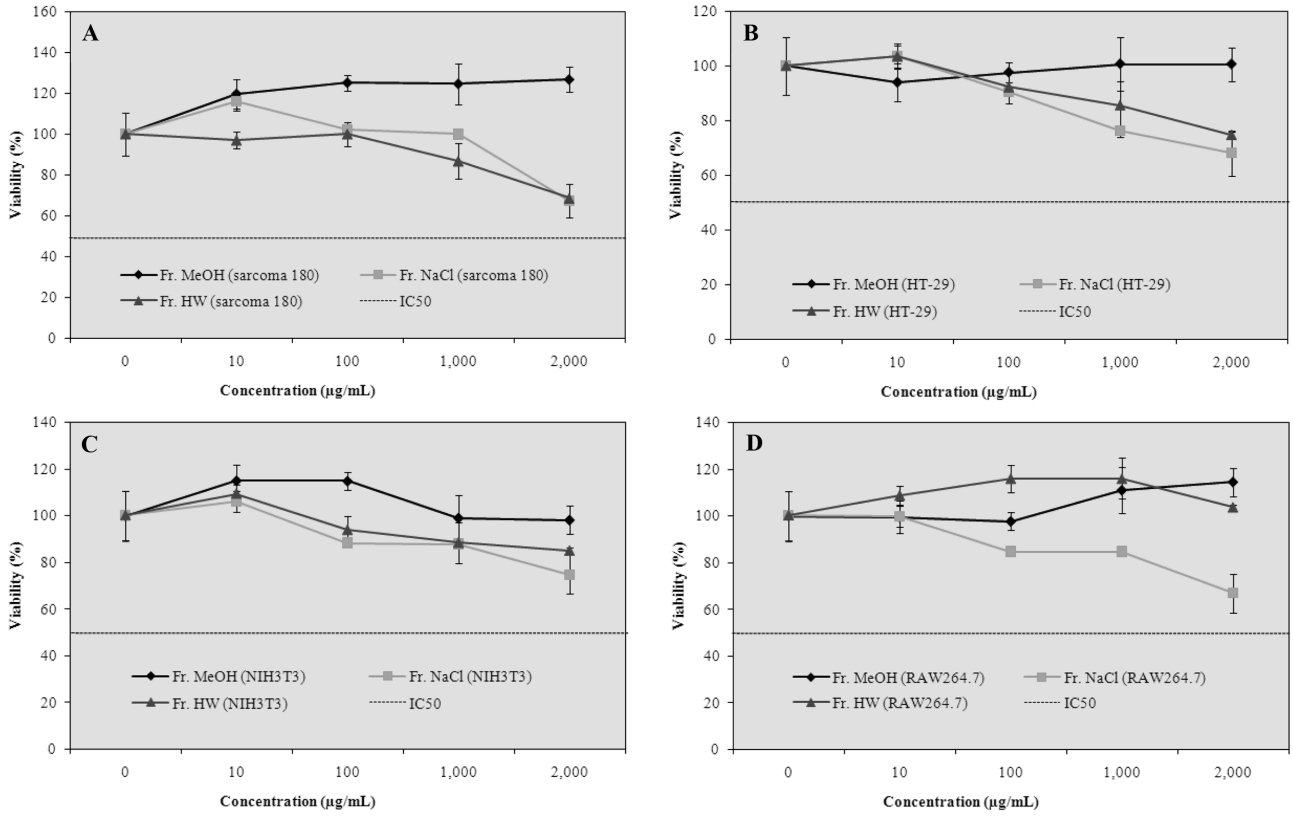

Fig. 1. *In vitro* cytotoxicity activity against sarcoma 180 (A), HT-29 (B), NIH3T3 (C), and D-RAW 264.7 (D) at different concentrations of various fractions extracted from *Pleurotus nebrodensis*. Values are expressed as mean ± SD (n = 5). Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl, fractions extracted with 0.9% NaCl solution; Fr. HW, fractions extracted with hot water.

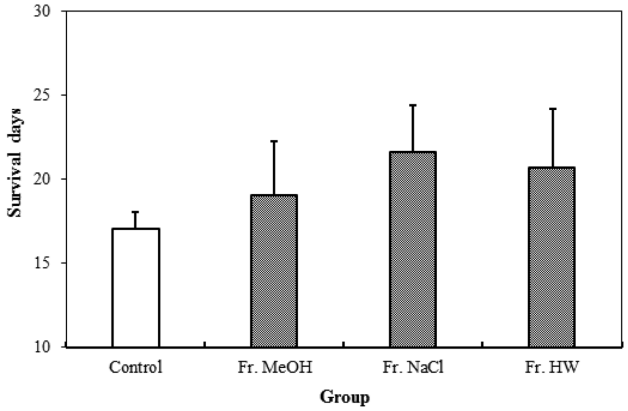

Fig. 2. Effect of various fractions extracted from *Pleurotus nebrodensis* on the life span of ICR mice inoculated with sarcoma 180. Values are expressed as mean ± SD (n = 10). Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl, fractions extracted with 0.9% NaCl solution; Fr. HW, fractions extracted with hot water.

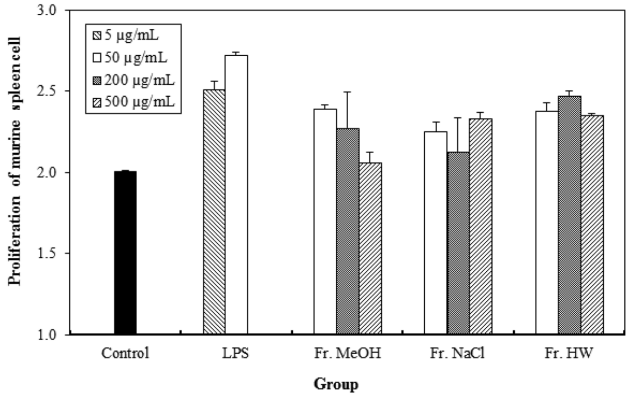

Fig. 3. Effect of various fractions extracted from *Pleurotus nebrodensis* on proliferation of murine spleen cells. Values are expressed as mean ± SD (n = 5). LPS, lipopolysaccharide used as a positive control; Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl, fractions extracted with 0.9% NaCl solution; Fr. HW, fractions extracted with hot water.

Fr. HW at 50 μg/mL resulted in an increase of APase activities of 1.48-, 1.22-, 1.39-, and 1.25-fold, respectively, compared to control (Fig. 4). APase activity in murine spleen cells showed a significant increase in Fr. HW at 500 μg/mL, compared with control.

NO production activity of fractions. NO production activity in the culture supernatants of RAW 264.7

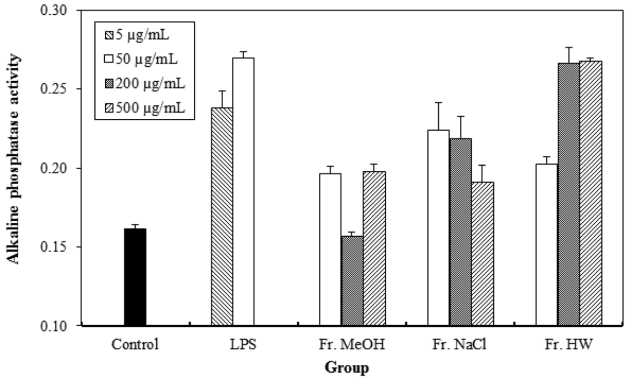

Fig. 4. Effect of various fractions extracted from *Pleurotus nebrodensis* on alkaline phosphatase activity in murine spleen cells. Values are expressed as mean ± SD (n = 5). LPS, lipopolysaccharide was used as a positive control; Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl; fractions extracted with 0.9% NaCl solution; Fr. HW, fractions extracted with hot water.

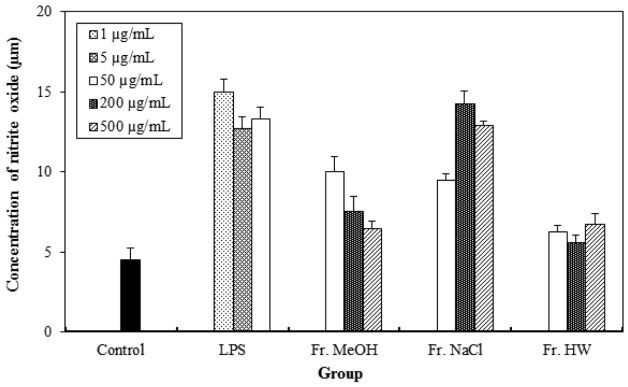

Fig. 5. Effect of various fractions extracted from *Pleurotus nebrodensis* on nitric oxide production in RAW 264.7. Values are expressed as mean ± SD (n = 5). LPS, lipopolysaccharide was used as a positive control; Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl, fractions extracted with 0.9% NaCl solution; Fr HW, fractions extracted with hot water.

macrophages with various concentrations (50, 200, and 500 μg/mL) of Fr. MeOH, Fr. NaCl, and Fr. HW fractions of *P. nebrodensis* ranged from 6.5~10.0, 9.5~14.3, and 5.6~6.8 μM, respectively. In the control group, 4.5 μM of NO was released, while 15.0, 12.7, and 13.3 μM of NO were produced by treatment with LPS at the concentrations of 1, 5, and 50 μg/mL (Fig. 5).

Effect of fractions on cytokine production. Results for production of cytokines in three different fractions of *P. nebrodensis* are shown in Fig. 6. At 10~1,000 μg/mL of Fr. MeOH, Fr. NaCl, and Fr. HW fractions on production of TNF-α, IL-1β, and IL-6, ranged from 37.16~53.90, 94.81~107.22, and 95.00~109.63 pg/mL, respectively (Fig. 6A), 62.12~82.85, 50.79~85.64, and 34.07~70.03 pg/mL, respectively (Fig. 6B), and 117.69~191.30, 166.98~224.05, and 175.43~460.42 pg/mL, respectively (Fig. 6C). The results indicated that production of TNF-α, IL-1β, and IL-6 was significantly higher, compared to control, and IL-6 production was excellent in contrast to TNF-α, IL-1β, and positive control, Con A, at the tested concentration of the various fractions.

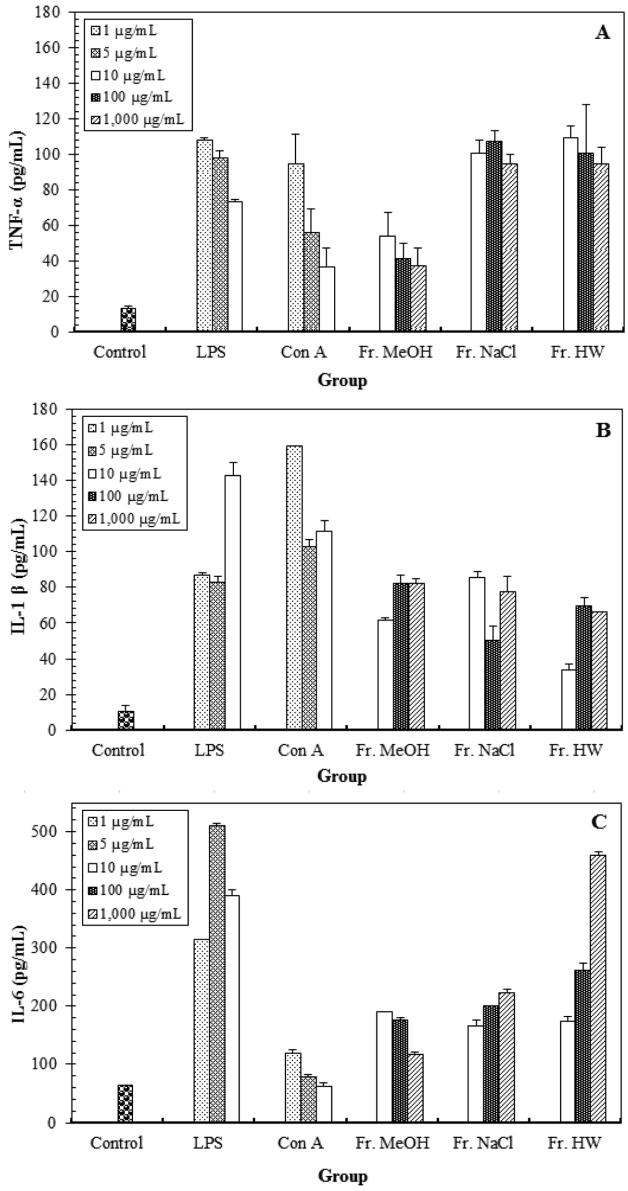

Fig. 6. Effect of different concentrations of various fractions extracted from *Pleurotus nebrodensis* on tumor necrosis factor-α (TNF-α) (A), interleukin-1β (IL-1β) (B), and interleukin-6 (IL-6) (C) production. Values are expressed as mean ± SD (n = 5). LPS, lipopolysaccharide used for positive control; Con A, concanavalin used for positive control; Fr. MeOH, fractions extracted with 80% methanol; Fr. NaCl, fractions extracted with 0.9% NaCl solution; Fr HW, fractions extracted with hot water.

Discussion

The immunomodulating effects of β-glucans are well established during development of immune reactions. Due to their immunomodulatory and antitumor effects, β-glucans and glucan-protein complexes isolated from mushrooms have been used as a source of therapeutic agents [15]. β-Glucan and protein contents in Fr. MeOH, Fr. NaCl, and Fr. HW extracts of *P. nebrodensis* ranged from 23.79~36.63 g/100 g and 4.45~6.12 g/100 g, respectively. Several investigators have isolated and purified immunomodulating polysaccharides from mushrooms as a modifier of biological response [16]. The anti-tumor activities of polysaccharides resulted primarily from their immunopotentiation effects [1, 2].

In vitro cytotoxicity assays can be used for prediction of toxicity for general screening of chemicals [17]. In determination of *in vitro* cytotoxicity activities of the three different fractions of *P. nebrodensis* against sarcoma 180, HT-29, NIH3T3, and RAW 264.7 cells, the results indicated that Fr. MeOH, Fr. NaCl, and Fr. HW fractions had no significant cytotoxic effects on four cell lines at the tested concentrations. In our earlier study, hot water extract from the fruiting body of *Elfvingia applanata* did not inhibit proliferation of HT-29, Hep G2, TR, and sarcoma 180 cancer cells [18]. In another study, Lee *et al*. [19] reported that a hot-water extract of *Inonotus obliquus* exerted little inhibitory activity against proliferation of human colon cancer cells, showing good agreement with our results.

Shim *et al*. [20] reported that treatment of sarcoma 180 tumor cells with methanol extract of *Paecilomyces sinclairii* resulted in little inhibition of tumor cell growth and prolongation of the life span of mice by 32.3%. In general, the criteria for judging the antitumor effect of any substance include prolongation of the life span by more than 25% [21]. This observation is consistent with our results showing a significant increase in the mean life span of the group treated with Fr. NaCl. It might be concluded that polysaccharides of *P. nebrodensis* have a good anticancer effect.

The results of this study suggested that β-glucan of *P. nebrodensis* can improve the immune response of the host via stimulating proliferation of immune organs, murine spleen cells. Li *et al*. [22] reported that proteoglycan extracted from crude liquid culture medium and mycelia of *Phellinus nigricans* stimulated proliferation of lymphocytes of spleen cells and also increased production of TNF-α and interleukins. Murine spleen cells are the primary residence of various immune cells and are also important for host immune response.

APase activity in murine spleen cells showed a significant increase following treatment with Fr. HW 200 μg/mL, compared with control. Kim *et al*. [23] reported an increase in APase activity by 1.3~1.6-folds upon stimulation with crude polysaccharides extracted from the fruiting body of *Grifola frondosa* at concentrations of 50~200 μg/mL. Therefore, it is concluded that treatment with Fr. HW could result in improved immunostimulating activity of the host via increasing APase activity. The results of this study demonstrated that β-glucan of *P. nebrodensis* can induce an increase in production of NO and can improve the immune response in ICR mice. Our results are similar to those of Kim *et al*. [24], who reported a dose-dependent increase in production of NO in RAW 264.7 macrophages stimulated by polysaccharides extracted from *Phellinus linteus*. Ooi and Liu [15] reported that polysaccharides extracted from mushrooms exert anti-tumor effects through activation of different immune responses in the host rather than by direct killing of tumor cells.

TNF-α, Il-1 β, and IL-6 are important regulators of host defense against tumor cells [25]. The observed increase in production of cytokines would suggest an enhanced ability of the host to combat the growth of tumors. Macrophages can be activated by β-glucans and other cell mediators to kill tumor cells by production of TNF-α and interleukins. The bioactivities of polysaccharides and polysaccharide-protein complexes are dependent on binding on the surface receptor of immune cells. These receptors, known as pattern recognition molecules, can recognize foreign ligands during the initial phases of the immune response [26]. Specifically, macrophages might bind polysaccharides via toll-like receptor 4, CD14, complement receptor 3, scavenger receptor, dectin-1, and mannose receptor [27]. Our results are in agreement with those reported by Ooi and Liu [15], showing that polysaccharides from mushrooms exert antitumor effects via activation of different immune responses in the host rather than by directly attacking cancer cells. Indeed, in our study, we show that mushroom polysaccharides trigger macrophages to produce varying levels of TNF-α, IL-β, and IL-6. Even among fractions and different concentrations of polysaccharide, the levels of cytokine release are different. These differences may reflect structural and conformational variations, as well as bioavailability of polysaccharides from these extracts.

On the basis of the results, it is suggested that the three different fractions of *P. nebrodensis* evaluated here could be used as an easily accessible source of immune potentiating polysaccharides that induced a significant reduction in tumor growth. Conversely, conduct of additional studies will be needed in order to elucidate the structural features and structure-function relationships involved in immunomodulation.

Acknowledgements

This study was supported by a mutual grant from the Rural Development Administration (Agenda 9-27-63; No.

200901OFT092763229), a research grant from the Korea National Research Resource Center Program through the National Research Foundation (NRF) through the Culture Collection and DNA Bank of Mushrooms (CCDBM), Division of Life Sciences, University of Incheon, Republic of Korea, and a research grant from the Korea National Research Foundation (2011-0016999).

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