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

Youn Jeong Cha¹, Nuhu Alam¹, Jae Seong Lee¹, Kyung Rim Lee¹, Mi Ja Shim¹, Min Woong Lee⁴, Hye Young Kim¹, Pyung Gyun Shin¹, Jong Chun Cheong⁶, Young Bok Yoo⁵ and Tae Soo Lee¹,²,³,⁴,⁶

¹Division of Life Sciences, University of Incheon, Incheon 406-840, Korea  
²Department of Botany, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh  
³Department of Life Science, University of Seoul, Seoul 130-743, Korea  
⁴Department of Life Science, Dongguk University, Seoul 100-715, Korea  
⁵Department of Clinical Laboratory Science, Shinsung University, Danggin 343-861, Korea  
⁶Mushroom Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumsung 369-873, Korea

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 point of view, extensive studies have been conducted for investigation of polysaccharides extracted from mushrooms.

Pleurotus nebrodensis, known as Bailingu oyster
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 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 xg 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 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 xg. 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 xg 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 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 µg/mL), and 10% fetal bovine serum (FBS) at 37°C with 5% atmospheric CO2 in a humidified incubator. Sarcoma 180 cells were maintained in ascitic form by serial transplantation every seven days in a male ICR mouse.
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 to measure cell viability and proliferation. Briefly, for the MTT assay, 100 µL of cells of HT-29, NIH3T3, and RAW 264.7 (10^4 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 CO₂. Thereafter, 10 µL of 5 mg/mL of MTT solution was added, followed by incubation at 37°C with 5% atmospheric CO₂ 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 cytoxicity 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 CO₂. Then, 1 mg/mL of 2,3-bis(2-methoxyl-4-nitro-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 CO₂ 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 × 10⁵ 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.

\[
\text{ILS (\%)} = \left[\frac{(T - C)}{C}\right] \times 100
\]

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 × 10⁶ 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 CO₂ 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 CO₂ 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 × 10⁶ cells/well), followed by incubation for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO₂. LPS at 5 and 50 µg/mL was applied to 100 µL of spleen cells (1 × 10⁶ cells/well), followed by incubation for 48 hr in 96-well microplates at 37°C with 5% atmospheric CO₂. 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 MgCl₂ (1 mM) to 10 µL of the cell lysate. The reaction mixture was incubated for 1 hr at 37°C with 5% atmospheric CO₂ 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).

\[
\text{SI} = \frac{\text{mean OD in the treated group}}{\text{mean OD in control group}}
\]

**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 ×
10^6 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 CO₂. LPS, as the positive control, was applied to 100 µL of RAW 264.7 cells (1 × 10⁶ 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 CO₂. Then, an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylendiamine 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 x 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 CO₂ 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 × 10⁶ 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 CO₂ 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 immunosorbent 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 ≤ 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). The protein level in Fr. NaCl was significantly higher (p ≤ 0.05), compared to Fr. MeOH and Fr. HW.

**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

| Fractions | β-Glucan (g/100g) | Protein (g/100g) |
|-----------|-------------------|-----------------|
| Fr. MeOH  | 36.63 ± 1.12¹     | 4.67 ± 0.56¹   |
| Fr. NaCl  | 23.79 ± 1.59¹     | 6.12 ± 0.26¹   |
| Fr. HW    | 29.37 ± 2.58¹     | 4.45 ± 1.21¹   |

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 ≤ 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.

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

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.
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 immunopotentiating 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 *Griifola 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.
References

1. Hsieh YS, Chien C, Liao SK, Liao SF, Hung WT, Yang WB, Lin CC, Cheng TJ, Chang CC, Fang JM, et al. Structure and bioactivity of the polysaccharides in medicinal plant Dendrobium huoshanense. Bioorg Med Chem 2008;16:6054-68.
2. Togola A, Innjerdingen M, Dall O, Barsett H, Rolstad B, Michaelsen TE, Paulsen BS. Polysaccharides with complement fixing and macrophage stimulation activity from Opilia celidifolia, isolation and partial characterisation. J Ethnopharmacol 2008;115:423-31.
3. Ladányi A, Tímár J, Lapis K. Effect of lentinan on macrophage cytotoxicity against metastatic tumor cells. Cancer Immunol Immunother 1993;36:123-6.
4. Tao Y, Zhang L, Cheung PC. Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides. Carbohydr Res 2006;341:2261-9.
5. Mueller A, Raptis J, Rice PJ, Kalbfleisch JH, Stout RD, Ensley HE, Browder W, Williams DL. The influence of glucan polymer structure and solution conformation on binding to (1→3)-β-D-glucan receptors in a human monocyte-like cell line. Glycobiology 2000;10:339-46.
6. Alam N, Shim MJ, Lee MW, Shin PG, Yoo YB, Lee TS. Phylogenetic relationship in different commercial strains of Pleurotus nebrodensis based on ITS sequence and RAPD. Mycobiology 2009;37:183-8.
7. Alam N, Yoon KN, Lee TS. Evaluation of the antioxidant and antityrosinase activities of three extracts from Pleurotus nebrodensis fruiting bodies Afr J Biotechnol 2011;10:2978-86.
8. Wang H, Ng TB. Eryngin, a novel antifungal peptide from fruiting bodies of the edible mushroom Pleurotus eryngii. Peptides 2004;25:1-5.
9. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
10. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
11. Geran RI, Greenberg NH, MacDonald MM, Schumacher AM, Abbot BJ. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep 1972;3:59-61.
12. Ngamwongsatit P, Banada PP, Panungrued W, Bhunia AK. WST-1 based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic Bacillus species using CHO cell line. J Microbiol Methods 2008;73:211-5.
13. Ohno N, Araizawa Y, Suzuki I, Yadomae T. Induction of alkaline phosphatase activity in murine spleen cells treated with various mitogens. J Phamacobiodyn 1986;9:593-9.
14. Choi SH, Jun CD, Lee BS, Park SD, Oh JS, Chung HT. Effect of various extrinsic and intrinsic factors on the nitric oxide production of murine macrophage. Korean J Biol Response Modif 1993;3:15-22.
15. Ooi VE, Liu F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Curr Med Chem 2000;7:715-29.
16. Mizuno M, Shiomi Y, Minato K, Kawakami S, Ashida H, Tsuchida H. Fucogalactan isolated from Sarcodon asperatus elicits release of tumor necrosis factor-α and nitric oxide from murine macrophages. Immunopharmacology 2000;46:113-21.
17. Scheers EM, Ekwall B, Dierickx PJ. In vitro long-term cytototoxicity testing of 27 MEIC chemicals on Hep G2 cells and comparison with acute human toxicity data. Toxicol In Vitro 2001;15:153-61.
18. Shim SM, Lee JS, Lee TS, Lee UY. Antitumor and immunostimulating activities of Elvingia aplanata hot water extract on sarcoma 180 tumor-bearing ICR mice. Mycobiology 2012;40:47-52.
19. Lee SH, Hwang HS, Yun JW. Antitumor activity of water extracts of a mushroom, Inomoton obliquus, against HT-29 human colon cancer cells. Phytother Res 2009;23:1784-9.
20. Shim SM, Im KH, Kim JW, Lee UY, Shim MJ, Lee MW, Lee TS. Studies on immuno-modulatory and antitumor effects of crude polysaccharides extracted from Paecilomyces sinus irritated. Kor J Mycol 2003;31:155-60.
21. Clarkson BD, Burchenal JH. Preliminary screening of antineoplastic drugs. Prog Clin Cancer 1965;1:625-9.
22. Li X, Jiao LL, Zhang X, Tian WM, Chen S, Zhang LP. Antitumor and immunomodulating activities of proteoglycans from mycelium of Phellinus nigricans and culture medium. Int Immunopharmacol 2008;8:909-15.
23. Kim JH, Cha YJ, Shim MJ, Lee MW, Lee TS. Immunostimulating and antitumor effects of crude polysaccharides extracted from fruiting body of Grifola frondosa. Kor J Mycol 2011;39:68-77.
24. Kim KY, Choi GS, Lee SH, Park YM. Acidic polysaccharide isolated from Phellinus linteus enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages. J Ethnopharmacol 2004;95:69-76.
25. Wells SM, Kew S, Yaqoub P, Wallace FA, Calder PC. Dietary glutamine enhances cytokine production by murine macrophages. Nutrition 1999;15:881-4.
26. Gordon S. Pattern recognition receptors: doubling up for the innate immune response. Cell 2002;111:927-30.
27. Brown GD, Gordon S. Immune recognition: a new receptor for β-glucans. Nature 2001;413:36-7.